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Age, Access, and Sweets-Motivation

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by

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Abstract

Availability can have profound influence on the consumption of foods and drinks. The 2-phase intermittent-continuous protocol (ICP) examines sucrose solution intake in two groups of rats and finds intermittent access significantly increases intake. In Phase I, rats receive intermittent or continuous access to a 4% sucrose solution, and with adults this results in a long-term elevation (a doubling) in the intermittent group. In Phase II, when rats are shifted to common sucrose schedule, this difference is maintained. Adult rats given 16% sucrose in Phase I do not differ in consumption, but in Phase II with 4% sucrose, an unexpressed elevation in the intermittent rats becomes evident. From my MSc work, it appeared pups were protected from the ICP associated intake elevation. I tested rats with sucrose solutions to explore how availability changes intake over age. First, intake of 4% sucrose was examined in a cross-sectional experiment that compared two or three intermittent exposures to sucrose (with continuous access) across three developmental periods (pups, adolescent, and adult) using weight corrected consumption. All rats increased intake over 2 intermittent exposures and decreased it with continuous access. Adolescent rats consumed more sucrose than pups and adults. I then tested pup and adult rats (in the ICP) with 4% or 16% in Phase I, with all receiving 4% in Phase II. In parallel ICP experiments with adult and pup rats, adults demonstrated the difference in Phase II with both sucrose concentrations while pups only developed the difference with the 16% solution, and when differences developed to 4% in Phase II, they remained latent until mid to late adolescence. I then tested pups with the ICP and

16% in Phase I, and included additional 10-day gap without sucrose for some groups (+Gap groups) between Phase I-II to examine the robustness of developing sucrose intake differences in younger rats. A very robust sucrose intake difference slowly emerged in Phase II, with the gap itself inducing an additional elevation in consumption (often called the elation effect) that was independent of the intermittent vs. continuous difference. The sucrose intake difference emerged slowly in both the non-gap and the +Gap groups when given access. Lastly, I examined how these access-induced sucrose differences relate to the brain's response by exploring sucrose intake-related Fos-expression and complimentary complex network analysis of the Fos-data. The ICP-Fos study identified the ventral pallidum, posterior part of the paraventricular thalamus, parts of the paraventricular hypothalamus, and the ventral part of the lateral septum as areas that are possibly involved with the sucrose intake differences that develop with the ICP, while the network analysis revealed some differences in functional connectivity that might be related to the behavioural differences with a complex developmental profile. The primary finding of this work was that sucrose availability can have a profound delayed influence on pups, and importantly, the potentially maladaptive behaviour of prolonged elevated sucrose intake can develop in pups but remains latent until later in life.

Keywords: Intermittent access; availability; sucrose; development; rats

Dedication

I would like to dedicate this work to my mother, Fatima Senthinathan, for her resolute support which gave me strength through the most difficult times.

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List of Abbreviations

ANOVA	Analysis of variance
BEP	Binge eating prone
BER	Binge eating resistant
BNST	Bed nucleus of the stria terminalis
DAB	3,3'-Diaminobenzidine tetrahydrochloride
DE	Deprivation effect
DTT	Dorsal tenia tecta
ED	Every day (access)
E2D	Every second day (access)
E3D	Every third day (access)
Fos	Protein product of the immediate early gene *c-fos*
FOS-IR	Fos-Immunoreactivity
IAE	Intermittent access effect
ICP	Intermittent-continuous protocol
IEG	Immediate-early gene
IP	Intra-peritoneal
IR	Immunoreactivity
LS	Lateral septum
M-W-F	Monday-Wednesday-Friday protocol
NAc	Nucleus accumbens core
OEE	Over extinction event
PB	Phosphate buffer
PBS	Phosphate-buffered saline

PREE	partial-reinforcement-extinction-effect
PVH	Paraventricular hypothalamus
PVT	Paraventricular thalamus
pPVT	Posterior part of the paraventricular thalamus
VP	Ventral pallidum

Chapter 1: Introduction

Availability is a complex issue that impacts how food and drink are consumed. It is generally accepted that the homeostatic need prompts hunger and thirst to ensure the satisfaction of an organisms' energy, nutrient and other physiological requirements (Rosenzweig, 1986); however, the influence of availability outside of needs on short- and longer-term patterns of consumption is less clear. Changes in environmental conditions, including the types of food we eat, and the availability of these food sources (i.e. the food climate) contributes to patterns of food consumption (Kearney, 2010). Over the past half-century, humans have experienced an increased availability of low cost, high-calorie sugar sweetened beverages and other heavily processed highly palatable foods along with a concomitant trend of increased sugar intake (Malik et al., 2010). The positive correlation between increased sugar intake and health issues including diabetes, cardiovascular disease, and obesity have brought to forefront the importance of looking more fully at the control of sweet consumption (Johnson et al., 2007; Rippe & Angelopoulos, 2016).

To humans and other animals, sugary foods and drinks are typically 'innately highly' palatable (Berridge, 2004; Berridge & Pecina, 1995; Ganchrow et al., 1986; Steiner et al., 2001), so it may not be surprising that they are often overconsumed. An improved understanding of the circumstances that contribute to increased sugar consumption and the underlying changes that maintain elevated sugar consumption might be helpful.

Human and rat work show several age-related differences in sucrose intake levels (Langlois & Garriguet, 2011), taste sensitivity (Inui-Yamamoto et al. 2017), acceptability (Bertino & Wehmer, 1981) and preference (Desor & Beauchamp, 1987). For example, from childhood onwards increasing age is associated with reduced preference for sweets (Drewnowski, 1989; 2000). I was interested in exploring how the availability of sugar changes consumption across age. Using an intermittent access protocol to explore sugar consumption, I tested how availability influences the intake of sugar solutions in nondeprived rats at various developmental stages.

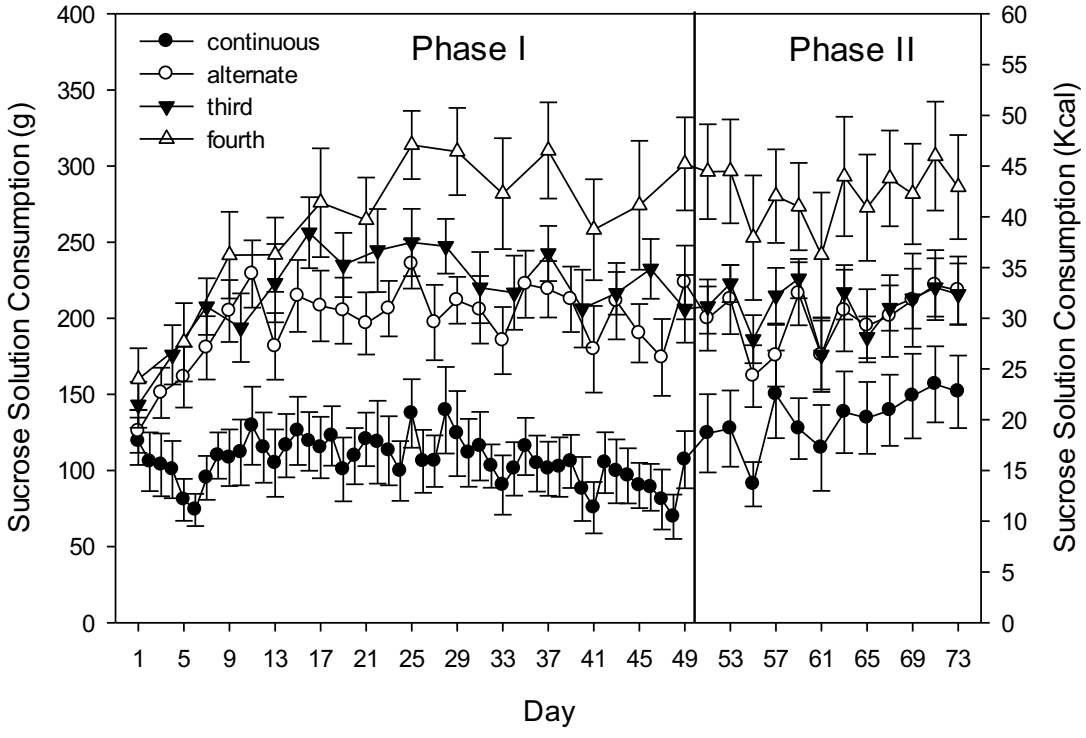
The format of this dissertation is as follows: Chapter 1 serves as an introduction to the experiments. Chapter 2 describes one large behavioural experiment testing rats at various developmental stages (as pups, adolescents, and adults). Chapter 3 presents a series of behavioural experiments with pups and adults. Chapter 4 explores work (mirroring some of the experiments in Chapter 3) designed to uncover neural structures and networks associated with differentiated sucrose consumption in pups and adults.

Replicated work from Eikelboom's lab has shown with the Intermittent vs. Continuous Protocol (ICP) groups of adult rats given intermittent access (24 h every third day) to a 4% sugar solution increase their intake of sucrose compared to groups with continuous daily access across several weeks (Celejewski, 2011; Eikelboom & Hewitt, 2016; Senthinathan, 2012). When these groups are shifted to a uniform sucrose availability schedule (every 2nd day access) in a second phase, intermittent groups continue to consume more sucrose than continuous

groups (Celejewski, 2011; Eikelboom & Hewitt, 2016; Senthinathan, 2012). This sucrose consumption difference caused by the initial availability between the groups (intermittent vs. continuous access) is robust and persistent among adult rats (see Figure 1.1 from Eikelboom & Hewitt, 2016).

Figure 1.1

Mean (± SEM) 4% sucrose intake in grams (left y-axis) and in Kcal (right y-axis) for adult rats receiving solution intermittently (every alternate, third, or fourth day) vs. continuously in Phase I (49 days). All groups had alternate day access to 4% in Phase II (24 days) (from Eikelboom & Hewitt, 2016).



I tested young rats with the ICP in my master's work and found the effect of availability on sucrose consumption was not apparent in pups (Senthinathan, 2012). To follow up on this work, I explored the relationship between age, availability, and sucrose consumption by testing rats at different stages of development in several experiments that are described in this dissertation. Age, availability, as well as the concentration of a sugar solution can influence patterns of sugar consumption, therefore these variables must be considered (Bertino & Wehmer, 1981; Senthinathan, 2012).

Because I was particularly interested in exploring how developmental stage influences access-related changes in sucrose consumption, I tested rats at various ages from pups to young adults. The experiments in this dissertation involve groups of nondeprived rats differentiated by patterns of sucrose availability. The profound impact of intermittent access on sugar consumption is central to this work. To frame this in this chapter, the literature relating to the evolving of feeding, age, availability, and sugar consumption, is described, with the aim of bringing them to an intersection before the experiments proper.

The Regulation of Feeding, Age, and Growth

Homeostatic and hedonic processes regulate the control of feeding in concert. Homeostatic hunger and feeding serve to maintain energy balance. A depletion of energy results in an increased drive to eat via homeostatic processes. Hedonic hunger and hedonic feeding reflect the drive to obtain pleasure from feeding in the absence of an energy deficit (Lutter & Nestler,

2009). This reward-related feeding can augment homeostatic processes by increasing the motivation to over-eat foods that are highly palatable.

Typically feeding and drinking are discussed in terms of homeostasis (regulatory feeding and drinking), and this type of consummatory behaviour has received considerable attention (Casanova et al., 2019; Suzuki et al., 2011). Homeostatic feeding is conventionally described using a drive-reduction framework whereby a disruption of homeostasis in the body (e.g. lack of nutrition) triggers a need state (e.g. hunger) leading to efforts to obtain food. Homeostatic feeding is supported by short- and long-term mechanisms that regulate energy intake. For example, seeing food, its taste, consumption, and digestion processes increase the production of short-term hormonal signals such as ghrelin and cholecystokinin (CCK) that increase, or decrease feeding, respectively. Longer-term regulation of feeding is supported by the protein hormone leptin, which is produced and secreted by adipose cells. Leptin-signaling by afferent projections to the arcuate nucleus of the hypothalamus provides a long-term index of over- or under-eating (Casanova et al., 2019). Together, short- and long-term hormonal signals serve to maintain a fairly constant energy balance.

Consumption that is not essential for an animal's survival is likely shaped by experience with highly rewarding food sources. Hedonic (non-regulatory) feeding/drinking has been discussed using an incentive motivational framework involving positive reinforcement and wanting, an active process that attracts a subject towards a stimulus in search of affective reward (Berridge, 2007; 2019).

Circulating hormones (leptin and ghrelin) known to be involved in homoeostatic feeding also exert influence on hedonic feeding in both rats and humans (Edwards & Abizaid, 2016; Farooqi et al., 2007; Fulton et al., 2006, Jerlhag et al., 2007; Malik et al., 2008).

The drive reduction model associated with the homeostatic control of feeding can be integrated into an incentive approach that supports hedonic feeding by assuming that need changes the incentive value of an appropriate stimulus. Perhaps the best way to conceptualize feeding generally is under an incentive motivation framework involving an innate reward system where need elevates the incentive value of food (Berridge & Robinson, 2016; Cabanac, 1971; Toates, 1986; Lutter & Nestler, 2009).

Mammalian Feeding Behaviour

Mammalian feeding and optimal feeding strategies can vary widely between species. Differences in feeding including the types of foods consumed and patterns of food consumption are likely shaped by environmental pressures, evidenced by a diversity of feeding practices among mammals from seemingly continuous small leaf consumption among ungulates to whole animal intake typical of larger predators. The diversity of mammalian feeding supports a range of specific dietary needs among disparate species that vary in their need for fats, proteins, carbohydrates, and other nutrients. Although the adult mammalian diet shows such diversity, immediately post-natal, all mammals are seemingly dependent upon nutrition from their mother’s milk for some period of time. Weaning or the process of decreasing suckling and increasing consumption of

liquid and solid nutriment from other sources is an integral part of early development across mammalian species, and feeding continues to change with age.

Feeding Behaviour and Development in Rats

The age of weaning varies among species. Laboratory rats are often removed from their mother's cage (weaned) at about 21 days of age. At this age rat pups normally begin to consume nutriment from other sources while reducing suckling thereby lessening consumption of their mother`s milk (Thiels et al., 1990).

Rats are extremely flexible with regard to nutrition sources. This flexibility has likely contributed to their survival in the wild and their use as laboratory animals in feeding studies (Barnett, 1976). Like other mammals, rats consume a regulated number of calories and maintain a relatively constant amount of usable energy. In the lab and in nature, rats with access to multiple food sources do not typically restrict themselves to a single food source. When multiple food sources are available, rats will usually sample all, the proportion of each food consumed by a rat can reflect the palatability and caloric value of the food. Beginning with very young pups, the following subsection describes age-related differences in feeding over the rat lifespan.

Feeding, body growth, and weight gain are closely related (Harte et al., 1948). Rats use taste and post-ingestive signals to regulate feeding and maintain a constant intake of usable energy. Food consumption and body weight are

intertwined; rats grow larger and increase in weight up to adulthood and food consumption increases with age, at least until this period. Once they reach adulthood further weight gain is mainly related to accumulation of body-fat (in the Chapter 2 experiment, because I am comparing daily sucrose intake in rats at different ages/sizes, and the age/size of the rats impacts how much they can consume in a day, much of this analysis compares consumption per 100 g of body-weight to equalize groups, rather than raw sucrose solution intake).

Early in development, changes in feeding and growth are easily observable as young rats rapidly grow in size and weight. Consumption becomes more adult-like as rats mature and growth rate slows. The relationship between rat feeding and growth can be separated into three age-dependent growth phases, and the impact of feeding on body-growth varies across these phases.

Pups (Birth to Puberty). Immediately after birth, rats show a rapid increase in weight that is accompanied by an increase in body size (length from nose to anus) (Pitts, 1984). The Pup period spans from birth to about 35 days of age and can be subdivided by behavioral change across the pup period (Thiels et al.,1990). The youngest rats tested in the experiments described in this dissertation were about 22 days old.

18-26 days of age: By about 18 days of age rats initiate food intake and subsequently begin drinking water in the following days. Non-feeding behaviours including social grooming and play-fighting also markedly increase across this period (Meaney & Stewart 1981a; 1981b). This general trend of increased

behavioural activity has previously been described as restlessness or curiosity to explore outside of the nest (Bolles & Woods, 1964; Small, 1899). As noted earlier, laboratory rats are typically weaned at about 21 days of age. In the wild, at this age pups may venture away from the nest and their maternal food source, but leaving the nest is more commonly associated with the latter end of the pup period, and adolescence. In my experiments with pups in this dissertation, pups were 21-22 days old at the beginning of each experiment.

27-35 days of age: This latter part of the Pup period represents a transition to more adult-like patterns of activity. During this part of the Pup period, energy intake by pups increases and peaks compared to any other point in the rat lifespan (intake measured in this case as a function of total surface area of the rat) (Harte et al., 1948). The reason for this age-related peak in energy consumption at the latter end of the Pup period is not clear. Differences in basal metabolic rate may account for the spike of energy intake by older pups and contribute to the age-related difference in caloric intake as a function of body surface area.

Prior to about 18 days of age pups are awake and active in the light or day cycle, which is related to food availability. By about 25 days of age pups begin to shift their pattern of activity to reflect that of a nocturnal animal. It should be noted that these changes are not discrete but happen gradually over time thus there may be considerable overlap among the three Pup phases described above. Perhaps the most striking age-related differences in consummatory behaviour are evident when testing rats around the time of puberty, about 35

days of age in the rat (Dalton-Jez, 2006; Swithers et al., 2004). These age-related changes that are described below seem to highlight the Pup period as a unique developmental period during which rats might be set to consume maximal amounts of energy.

Adolescents (Puberty to Adulthood). The beginning of adolescence coincides with puberty, the discrete ontogenetic change in psychological and neuroendocrine functionality associated with sexual maturation. Adolescence is a gradual transition period spanning from about 35-60 days of age when rats reach adulthood (Spear & Brake, 1983; Spear, 2000) (note, some disagreement exists as to whether adolescence is unique to humans). Some researchers assert in rats the onset of puberty is typically around day 32 (Ojeda & Urbanski, 1994) however the exact timing is disputed and may depend on growth rate (Kennedy & Mitra, 1963). Once a rat enters puberty it continues to grow in both length and weight until late adolescence (~41-54 days of age in male Sprague-Dawleys) when rats typically reach their adult size in length (Gabriel et al., 1992).

Adults (Adulthood Onwards). By ~60 days of age rats have grown to their full body size. The effect of increasing food consumption with increased age is attenuated in the adult rat because of a slowing rate of growth. Although adult rats continue to gain weight throughout their lifespan, this continued weight gain is mainly due to an accumulation of adipose tissue. Relatedly, the amount of food needed per unit of body weight decreases with increasing body weight.

Focus on Pups: Why Pups Might be Different

During the Pup period, free-fed rats consume more food and use more energy than older rats (Harte et al., 1948). From an evolutionary perspective a mechanism that supports maximal nutrient intake during development is adaptive, especially during times of food scarcity, because during development the brain is more vulnerable to disruption from malnutrition than the adult brain (Rosenzweig & Bennett, 1996).

Adult rats given an intraperitoneal (IP) injection of the fatty acid oxidation inhibitor 2-Mercaptoacetate (MA) increase food intake, but this procedure does not stimulate eating in pups (Swithers et al., 2004). It is conceivable that this failure to induce feeding by MA is due to developmental mechanisms that support maximal nutriment intake in very young rats. It seems that the regulation of feeding is less susceptible to intervention during the Pup period, suggesting that feeding may be maximized in younger animals. Presumably this effect may be rooted in evolutionary adaptation. Thus, it seems difficult to increase feeding during the Pup period. When adult rats given *ad libitum* (*ad lib*) food and water are provided with a running-wheel, they increase running and reduce caloric intake for several days (Afonso & Eikelboom, 2003). This perverse coupling of increasing caloric expenditure and declining caloric intake results in reduced body weight compared to control animals. Subsequently rats increase their caloric intake to match their energy expenditure but because of the initial weight loss rats continue to maintain a lower body weight. Though this effect has been robustly demonstrated in rats during the Adult period, the wheel-induced feeding

suppression is not evident during the Pup period (Dalton-Jez, 2006). Taken together, interventions that reliably increase, or decrease feeding in adult rats don't have the same predictable effects in pups and these findings seems to support the argument that feeding during the Pup period is regulated by unique developmental mechanisms.

Klump and colleagues examined the emergence of binge-eating over development in rats (Klump et al. 2011). Binge eating prone (BEP) and binge eating resistant (BER) female Sprague-Dawley rats observed across development showed differing binge-eating proneness (a tendency to consistently consume relatively large amounts of highly-palatable food such as high-fat, or high-sugar food in a short period). This difference was not evident during the Pup period, but gradually emerged during adolescence. This finding suggests biological involvement for binge-eating and consummatory behaviour and supports the argument that during the Pup period rats may be consuming foods at a maximal rate. The failure to demonstrate differences in feeding among BEP and BER rats in the Pup period may be due to a ceiling effect whereby BEP rats cannot increase consumption beyond that of BER rats, but when consumption became stable among BER rats, a consumption difference became apparent. Parallel to the finding in rats by Klump and colleagues, binge-eating

and the diagnosis of bulimia nervosa in humans is not typically observed before adolescence (Lock, 2010)[1].

Developmental factors that maximize nutritive consumption during the Pup period could increase the chances of an organism's normal development and survival to adulthood. Some evidence suggests that during the Pup period rats are "set to consume" calories at a maximal rate whenever a food source is available in order to prevent lack of nutrition at times of food scarcity (Spear, 2000). The age-dependent binging expression in BEP and BER phenotypes (Klump et al., 2011) and failure to increase (Swithers et al., 2004) or decrease (Dalton-Jez, 2006) feeding with procedures that reliably work in older rats suggests age-related developmental factors may exist in very young rats that are not maintained in older rats. Like much of this previous work showing various feeding related phenomena (e.g. wheel induced feeding suppression, binge-eating proneness) typically present over adolescence (but not in pups), I found a similar pup effect (or lack thereof) with the ICP. With adult rats, we typically find a large sucrose intake difference between rats receiving 4% sucrose every third day vs. every day (Figure 1.1). With young rats, sucrose consumption between rats receiving 4% sucrose every third day vs. every day is similar in pups and an

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[1] As an added parallel, like women with bulimia, rats that binge-eat tend to be of normal weight and do not differ in rate of diet-induced obesity when compared to rats resistant to the development of binging behaviour (Boggiano et al., 2007; Oswald et al., 2011).

intake difference between rats receiving 4% sucrose every third day vs. every day only very gradually emerged over later adolescence (Senthinathan, 2012).

The relationship between age, intermittent access, and sugar consumption warrants further consideration. Will the previously defined developmental periods (i.e. Pup, Adolescent, Adult) show age-related differences in how the sugar intake is regulated and impacted by intermittent access? With a sequential design, I tested the influence of age or development stage on sucrose consumption (Chapter 2). Additionally, I used the ICP and explored age-related differences in sucrose consumption (Chapter 3) and related neural activity (Chapter 4).

Availability Influences Consumption

The profound impact of intermittent access on sugar consumption is central to my work. Seminal investigations exploring the relationship between access and intake strongly impacted subsequent work on food, drink, drug consumption, and other reward-related behaviours (Sinclair & Senter, 1967; 1968). These early works contributed to our understanding of access-induced changes in consumption. The earliest of these demonstrations was done with ethanol (i.e. alcohol or “drinking alcohol”).

Studies exploring the consumption of various solutions have demonstrated that simple access manipulations can have a profound impact on the subsequent intake of a given solution (Avena et al., 2008; Corwin & Wojnicki, 2006; Eikelboom & Hewitt, 2016; Sinclair & Senter, 1967; 1968). Sinclair and Senter

(1967) provided rats with *ad lib* or continuous access to ethanol, food, and water for 4 weeks. Subsequently rats were assigned to two groups and given either continuous or intermittent weekly access (i.e. 7 days of continuous access followed by 7 days of forced abstinence repeatedly) to a 7% ethanol solution for 8 weeks. Quantifying ethanol preference, defined as the amount of ethanol consumed daily by each rat divided by the total amount of liquid consumed by each rat, the authors demonstrated a transient increased preference for the ethanol solution over water among rats only in the intermittent group (Sinclair & Senter, 1967). On the day after each of the four weekly periods of ethanol deprivation the rats in the intermittent group significantly increased their intake of ethanol. This transient increase in voluntary drinking of ethanol induced by a single period of forced abstinence was termed the alcohol deprivation effect (DE) (Sinclair & Senter, 1967). The increased intake or preference associated with the alcohol DE was followed by a gradual decline in ethanol drinking over the following 6 days when ethanol was provided continuously, eventually reaching consumption levels comparable to rats with continuous access to the solution (Sinclair & Senter, 1967). Thus, the impact of restriction (deprivation) on subsequent consumption of a given solution is influenced by an animal's experience or history of consumption with the solution.

In Sinclair and Senter's (1967) work in which the alcohol DE was first observed, all rats were provided with 4 weeks of continuous access prior to the first deprivation period. To trace the development of the alcohol DE, Sinclair and Senter (1968) gave 4 groups of alcohol naïve rats a 7% ethanol solution for 1, 7,

or 21 days of an ethanol pre-exposure period followed by 6 days of ethanol deprivation and a subsequent 6-day post-deprivation period during which the ethanol solution was again made available. A control group had continuous access to the ethanol solution throughout the experiment. Immediately following the deprivation period, a significant alcohol DE was evident among the 21-day pre-exposure group and a similar but nonsignificant trend was evident among the 7-day pre-exposure group while rats in the 1-day pre-exposure group did not show an alcohol DE. Similar to their 1967 study, the ethanol DE was greatest immediately following the period of deprivation and gradually declined over the 6-day post-deprivation period (Sinclair & Senter, 1968). The way that ethanol solutions are consumed by rats likely involves an interaction among several factors including its novelty, availability, flavor, caloric value, and psychoactive properties.

Following the ethanol experiment, subsequent studies demonstrated a DE with the non-nutritive sweetener saccharin (Gandelman & Trowill, 1969, Pinel & Rovner 1977). The saccharin literature sometimes refers to the DE as the saccharin elation effect (Pinel & Rovner, 1977), but for simplicity it is referred to as the DE in this dissertation. The availability and flavour profile of a solution, as well as an animal's experience with the solution, and other solutions, can impact how it is consumed (Sinclair & Senter, 1968).

To test whether the length of the deprivation period impacts the magnitude of the alcohol DE, Sinclair and colleagues gave rats 40 days of continuous access to a 7% ethanol solution and subsequently subjected rats to ethanol

deprivation periods of 1.5 h, 1, 2, 5, 30, or 75 days. Over the first 30 days with continuous access to the ethanol solution the rats gradually increased their intake of ethanol; this initial acclimation reached a plateau during the final week prior to the implementation of the deprivation periods. The DE was not evident among rats deprived of access for less than two days. (Sinclair, Walker, & Jordan, 1973). With a 7% ethanol solution the DE increased rapidly up to 5 days and then became stable with only slightly increased ethanol intake induced by longer periods of deprivation.

Wise (1973) revisited this work to directly assess the impact of intermittent availability on consumption. Rats with intermittent 24 h every second day (i.e. alternate day) access to a 20% ethanol solution increased their intake and preference for ethanol over water while rats with continuous access did not show this pattern. Repeated intermittent exposure induced an increased preference for 20% ethanol, a concentration that rats normally find slightly aversive. This increased intake of a given solution induced by the repeated cycling of availability and restriction is referred to as the intermittent access effect (IAE).[2]

The generality of the IAE has been explored with non-psychoactive solutions including quinine, saccharin, citric acid, and salt (Wayner et al.,1972). Wayner and colleagues provided rats with continuous access to a mild 0.05%

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[2] Although the IAE and the DE are similar in that they both demonstrate the increased intake of a given solution following a period of forced abstinence, unpublished evidence from our lab suggests that there may be some important differences between the DE induced by a single gap and the IAE of increased intake induced by repeated intermittent exposure.

saccharin solution, food, and water, for 20 days and these nondeprived rats consumed minimal amounts of their daily fluid intake from the solution. Subsequently, rats were restricted from the saccharin solution on alternating days (for about 26 days or 13 intermittent days, varying slightly between animals), and these rats significantly increased their intake of the solution. To test the stability of the IAE, following this period of intermittent access the rats were given continuous access to the saccharin solution until the end of the experiment on Day 73 (Wayner et al.,1972). During this period of continuous access, rats showed varying rates of decline in consumption of the saccharin solution and increased water intake. Some rats continued to show a preference for the saccharin solution over water until the end of the experiment. The period of intermittent access induced a pattern of increased saccharin consumption by the rats, and the results showed access-induced changes in consumption manifest very quickly and can be resistant to change.

To investigate how intermittent access impacts the consumption of a mildly aversive fluid (thus similar to higher concentrations of ethanol) but devoid of calories and psychoactive properties, nondeprived rats were given continuous access to a mildly aversive 0.05% solution of quinine, which is a non-caloric bitter tastant. For 9 days rats had continuous access to the quinine solution but only consumed trivial amounts. Once it was established that the consumption remained low with this concentration of quinine, the solution was withdrawn for 2 days and subsequently made available on alternating days. When the rats were switched to this cyclic intermittent access schedule, they showed a marked

increase in consumption of the quinine solution. Similar to the results from Wise (1973) with 20% ethanol, repeated intermittent exposure to a mildly aversive, but in this case non-nutritive, solution induces an increased intake of the given solution (Wayner et al.,1972). For both quinine and ethanol, intermittent access seems to increase preference and/or consumption of an otherwise non-preferred solution.

The early work with alcohol (Sinclair & Senter, 1967; 1968; Wise 1973), saccharin (Gandelman & Trowill, 1969; Pinel & Rovner, 1977), and various other solutions (Wayner & Fraley, 1972; Wayner et al., 1972) clearly showed that deprivation or restricted availability can significantly increase subsequent intake or preference for a given solution. This increased intake induced by restricted availability is even evident when testing aversive or non-preferred solutions that rats typically avoid (Wayner et al., 1972; Wise, 1973). Following a period of restriction, the increased consumption or preference by rats for an otherwise non-preferred solution highlights the strength of simple access manipulations; however, it may be easier to demonstrate an increase in preference-aversion functions when the baseline intake levels are minimal (as with quinine, 0.05% saccharin, and high concentrations of ethanol).

The initial work by Sinclair and Senter (1967) that demonstrated the alcohol DE, and subsequent work by Wise and colleagues testing the influence of availability on consumption spurred a flurry of studies, and then until recently, this research was largely ignored. About 50 years after the initial observation of the alcohol DE and the IAE, intermittent schedules are again being used to

model and study alcohol abuse (Carnicella et al; 2014; Jeanblanc et al., 2019; Simms et al., 2008; Simon-O'Brien et al., 2015; Spoelder et al., 2017 a, b). Along with this revival in the intermittent alcohol work and likely because of concerns surrounding obesity and debate surrounding the concept of "sugar addiction", a similar resurgence has been seen in work on intermittent consumption of sweet solution (Avena et al. 2008; Eikelboom & Hewitt, 2016; Hoebel et al., 2009; Lenoir et al. 2007; Rehn & Boakes, 2019; Wiss et al. 2018).

Contrasting the bitter and aversive properties of quinine and ethanol, sweet solutions are innately preferred. This preference for sweeter tasting foods was likely shaped through our evolutionary past as sweet taste can serve as a gauge to caloric density, an important component of nutritive value. To understand how intermittent access to sweet solutions impacts their consumption it is important to consider how these solutions are consumed in *ad lib* (continuous) access conditions.

Continuous Access to Sweet Solutions

Sucrose

Not surprisingly, rats (and other omnivores) demonstrate an increasing preference for sweeter solutions. Given the choice between two solutions ranging from 1-64% sucrose concentration, rats reliably consume more of the sweeter solution in short term tests (Young & Greene, 1953). Longer two-bottle choice tests investigating the role of concentration on sucrose intake are difficult to interpret because of the satiety associated with consuming calories from sucrose.

Considering total volume consumed with single bottle access, which measures “acceptability" of solutions (Young & Green, 1953), in longer tests the sugar intake-concentration function takes on an inverted U shape. The declining volumetric intake for sugar solutions at higher concentrations seems to be driven by satiety or limits on caloric intake (Richter & Campbell, 1940; Collier & Bolles, 1968). When sugar drinking and actual consumption (ingestion into the stomach cavity) are parsed in the sham-drinking preparation, sugar intake increases with concentration (Mook et al., 1983). Given that satiety effects may make it difficult to interpret volumetric results from studies testing the intake of sucrose solutions it may be more informative to consider the amount of sugar solute rather than the volume of the solution that is consumed. Collier and Bolles (1968) maintained nondeprived rats with a 4, 8, 16, 32, or 64% sucrose solution over 40 days and reported that peak volume intake occurred for the 8% solution and was lower for lower and higher concentrations, creating an inverted-U, but solute intake peaked at a higher 16% concentration and then was stable at the high levels for the more concentrated 32 and 64% solutions creating a sigmoidal curve. This S-curve profile for sucrose solute consumption, and the connected, concentration-dependent inverted-U profile for sucrose volume consumption is highly reliable in adult rats (Sclafani & Nissenbaum, 1987; Smith & Sclafani, 2002; Spector & Smith, 1984; Smith & Wilson, 1989; Young, 1948). These patterns are fairly stable across the rat lifespan, with a shift to greater acceptance of very sweet solutions (Smith & Wilson, 1989) and complimentary increased preference for very sweet solutions related to decreased sucrose sensitivity at advanced ages

(Inui-Yamamoto et al. 2017). We are not aware of studies that have explored the volume-dependent sucrose intake curve or, or the calorie-limiting sucrose solute curve in pup, or adolescent rats.

Saccharin

With saccharin, satiety issues are avoided because it is devoid of calories; however, there are other complexities that must be considered. Given saccharin is an artificial sweetener, it should be expected that rats will readily consume saccharin as a preferred solution over water, but at very low, and very high concentrations, rats avoid saccharin solution. Saccharin intake increased as concentration increased to 0.1% and then decreased as concentration was raised to 0.3%, 0.9% and 2.7% (Smith & Sclafani, 2002). As with sucrose, the saccharin volume intake-concentration function forms an inverted U shape (Dess, 1993). For saccharin, lower concentrations are consumed less due to lack of palatability (lack of sweet taste). But, the descending portion of the inverted U for saccharin is likely due to saccharin's bitter after-taste that becomes more pronounced at higher concentrations, rather than satiety as with sucrose. With saccharin, this concentration dependent pattern (inverted U) for a 24-h period is the same if rats are sham fed (Sclafani & Nissenbaum, 1985), or tested in shorter protocols (Smith unpublished noted in Smith & Sclafani, 2002) or in preference tests (Smith & Rashotte, 1978).

Intermittent Access to Sweet Solutions

Recently, a few labs began to explore how intermittent access to sugar solutions (Avena et al., 2008; Eikelboom & Hewitt, 2016; Rehn & Boakes, 2019)

and other highly palatable foods (Corwin et al., 2011) impacts the consumption of these food sources. Two well-known limited or intermittent availability protocols are described below and followed by the relevant work from our lab. Notably, other than my MSc work described below, under “The Continuous vs. Intermittent Protocol (ICP)”, we are not aware of any other work exploring the influence of intermittent access on consumption that has tested developmental aspects.

12 h-12-h Protocol

Intermittent access protocols are defined by repeating periods of availability to a given substance followed by its restriction. One well established intermittent access protocol is defined by 12 h of sugar and food deprivation followed by 12-h access to food and a sugar solution (i.e. these rats are given intermittent access to sugar, but also food) and compared with various control groups including chow only, intermittent 12 h access to chow only, and most important to our discussion, continuous access to food and the same solution (Avena et al., 2008). In our understanding the Hoebel group has never tested nondeprived rats with intermittent 12-h daily sucrose. Experiments using this protocol have typically tested adult male Sprague-Dawley rats; 10% sucrose has been the most commonly used solution (Avena & Hoebel, 2003; Avena et al., 2008) but the Hoebel group have also used a 25% glucose solution (Colantuoni et al., 2001). Beginning 4 h into the dark cycle, rats in the intermittent sugar access group are given a 12 h period of sucrose and food availability. Subsequently, daily intake of the sugar solution is measured for all rats. After about 21 days of intermittent 12-h access to sugar, rats reliably consume a very

large sucrose meal at the beginning of the access period (i.e. a binge of sucrose drinking, defined as a short period of increased activity) followed by large but less frequent sucrose meals, compared to smaller but more frequent sucrose meals consumed by the rats with continuous access (Avena et al., 2008). With this protocol, no overall differences are evident when the total amount of sucrose consumed by both groups of rats on a given day is compared. Rats with intermittent 12-h access to a sugar solution drink as much of it during the 12 h period as those with 24 h or continuous access consume over the course of a day. Thus, with the 12 h-12 h protocol, intermittent access induces sucrose bingeing but does not necessarily induce increased sucrose consumption. An overall increase of sugar intake is evident if 12-h consumption by rats with intermittent access is compared to a comparable 12 h period for rats with continuous access because the sucrose-bingeing rats consume the sucrose solution at a faster rate across the 12 h of access.

Monday-Wednesday-Friday (M-W-F) Protocol

Hoebel's 12 h-12 h protocol involves a relatively short period of sucrose and food restriction followed by a period of sucrose availability. Another well-established intermittent access protocol, the M-W-F protocol, utilizes longer periods of restriction but shorter periods of access and does not involve any food deprivation. With the M-W-F protocol, the focus is on consumption patterns by an intermittent group that is provided with 2 h of access to a given substance on Monday, Wednesday, and Friday compared to the intake by rats with 2 h of daily access (the control group in the M-W-F protocol). Experiments that used the M-

W-F protocol have typically tested adult male Sprague-Dawley rats to explore how availability impacts the consumption of shortening (fat) (Corwin et al., 1998; Corwin, 2006). Sucrose, and high-fat diets have also been tested with this protocol (Corwin, Avena, Boggiano, 2011; Corwin & Wojnicki 2006). Similar to Hoebel's 12 h-12h protocol and the ICP (discussed in the next section), the M-W-F protocol compares highly-palatable food consumption between two groups but with this protocol both groups have limited access to the test substance.

Corwin and colleagues have shown that consumption by rats in their control group remains stable over the course of an experiment (Corwin & Wojnicki, 2006). After a few weeks of this procedure the rats given 2 h of access on Monday, Wednesday, and Friday, begin to show binge behaviour that is not evident in control rats (Corwin & Wojnicki, 2006). Notably, while rats with daily 2-h access to a palatable food or sugar solution consume stable moderate amounts across several weeks, those restricted to 2-h access on Monday, Wednesday, and Friday, escalate their intake. Binge-type behaviour is typically reported after about six weeks of this procedure. It is not clear why rats given 2-h access on M-W-F eventually escalate their intake of fats, while rats given 2-h daily access do not. One possibility is that rats are capable of tightly regulating food consumption behaviour across a 24 h period (controlled by a circadian oscillator) and rats have a harder time regulating intake across periods greater than 24 h. Regular daily 2 h availability can entrain to circadian rhythm in rats and result in changes associated with reward prediction, such as food anticipatory activity (FAA; daily increase in locomotor activity preceding the

presentation of food), which is regulated by a 24 h circadian oscillator, the suprachiasmatic nucleus of the hypothalamus (SCN). For example, the locomotor activity of rodents entrains to restricted food availability, and consequently, rodents become more active before food is presented (Richter, 1922). This might suggest predictability may be important in regulating consumption, particularly in the M-W-F protocol with 2-h access periods.

Although the rats with limited access on M-W-F develop binge behaviour and increased consumption (e.g. 2 h/day M-W-F rats consume two to three times more fat per day than daily 2 h/day rats), these rats gain weight at a comparable rate to the less restricted control rats that do not escalate their intake of the test substance. It appears animals that escalate fat intake reduce chow consumption. In terms of calories consumed, rats in the M-W-F group overeat on days when the test substance is available, however, this increased caloric intake is balanced by reduced caloric intake by these rats on days when fat is not available. Notably, rats with limited M-W-F access developed binge behaviour even when these rats did not under-eat on the previous day, suggesting rats did not know the restricted item was coming (Corwin, 2004). Thus, the increased consumption is not reflective of homeostatic need. Rats in the M-W-F group adjust their profile of fat intake due to their environment with sporadic availability.

The Intermittent vs. Continuous Protocol (ICP)

Adult rats provided with continuous access to a mildly sweet (4%) sucrose solution and *ad lib* food and water consume approximately 100-150 g of the

sucrose solution daily while rats given the solution intermittently every second, third, or fourth day show increasing intake of sucrose as the number of intervening days becomes greater; showing elevations up to about 300 g of solution a day (see Figure 1.1 above from Eikelboom & Hewitt, 2016). Some evidence suggests that intermittent gaps longer than 3 days may not result in further increased sucrose intake (McGee-Odger, 2013), possibly due to limits on fluid-volume consumption, caloric intake from sucrose, and their interaction. Replicated experiments show that rats given sucrose only every third or fourth day adjust their sucrose intake (all these experiments used 4% sucrose solutions) to about double the amount consumed by rats with continuous access (Eikelboom & Hewitt, 2016; Senthinathan, 2012; Celejewski, 2011). This increased intake of sucrose by rats intermittently given the sweet solution does not result in excess weight gain compared to rats provided with sucrose continuously or control animals with no access to sucrose because when consuming calories from sucrose solutions animals proportionately reduce their chow intake (Eikelboom & Hewitt, 2016). The number of calories consumed from sucrose totaled over multiple days by rats with continuous access to a 4% sucrose solution approaches the intake by rats with intermittent every third- or fourth-day access (Eikelboom & Hewitt, 2016; Senthinathan, 2012; Celejewski, 2011). To explore how availability impacts sucrose intake we have most frequently worked with adult male rats and a 4% sucrose solution because it provides strong hedonic value and with this protocol it reliably results in a large sucrose intake difference between rats with continuous or every day (ED) access

and rats with intermittent every third day (E3D) access, which is the typical Phase I effect (ICP has 2 distinct phases). A similar robust effect has been demonstrated with adult females (Celejewski, 2011).

In Phase I of the ICP, rats are given E3D vs. ED access to sucrose. After this period of differentiated access, which results in consumption differences, both groups are shifted to an identical E2D access schedule (Phase II). Therefore, both groups experience a 1-day shift in sucrose availability; the ED group begins receiving the solution less frequently (shifting from daily access to alternate day access) while the opposite occurs for E3D group. With adult rats given 4% sucrose in Phase I, the large sucrose consumption difference established in Phase I remained stable in a Phase II when all rats had E2D access (Eikelboom & Hewitt, 2016). Maintenance of the differentiated sucrose consumption behaviour during Phase II of the ICP is reflective of a persistent change in the animals due to their differing experience with sucrose (Phase I). The differentiated consumption appears very persistent, as it has been shown to last with saccharin, using a similar procedure, for more than 50 days of equal access (Celejewski, 2011).

For most experiments with Eikelboom's protocol we have utilized a Phase I of 10-15 cycles (Celejewski, 2011; Eikelboom & Hewitt; 2016; Senthinathan, 2012; Valyear, 2014). To test if the persistent differentiated consumption (Phase II effect) would be evident with a shorter duration of Phase I, Eikelboom and Hewitt (2016) gave rats 10 days of ED access to 4% sucrose or four intermittent E3D exposures in a relatively short Phase I before shifting both groups to Phase

II with E2D access. During Phase I rats with intermittent access quickly increased their intake of sucrose while rats with continuous access reduced their intake, resulting in a large (~100 g) sucrose intake difference whenever the sweet solution was available to both groups on common sucrose days. In Phase II, the E3D group stably maintained their elevated sucrose drinking. The group difference that was evident in Phase I was initially evident in Phase II but with continued E2D Phase II exposures the group difference disappeared because the ED group moved to alternate day access increased their intake of sucrose. Experiments with a longer Phase I (more sucrose exposures for continuous and intermittent groups) might produce more persistent differentiated sucrose consumption behaviour but this effect has not been systematically tested.

With the ICP, differentiated consumption of a sweet solution in Phase II highlights the stability of the change in sucrose consumption behaviour, or access induced-change caused by the experience of Phase I. Similar results have been obtained with the non-nutritive sweetener saccharin and various other sweet solutions, suggesting that for this phenomena the taste may be more important than caloric value (Celejewski, 2011; Rehn & Boakes, 2019).

Richter and Campbell (1940) demonstrated that the way sugar solutions are consumed is concentration-dependent. Work from our lab reliably demonstrates that when consumption by rats provided intermittent access to a 4% sugar solution is compared to intake by rats provided the solution continuously, a large consumption difference or IAE is evident. Various concentrations of sucrose solution have been tested in adult rats including 1-, 4-,

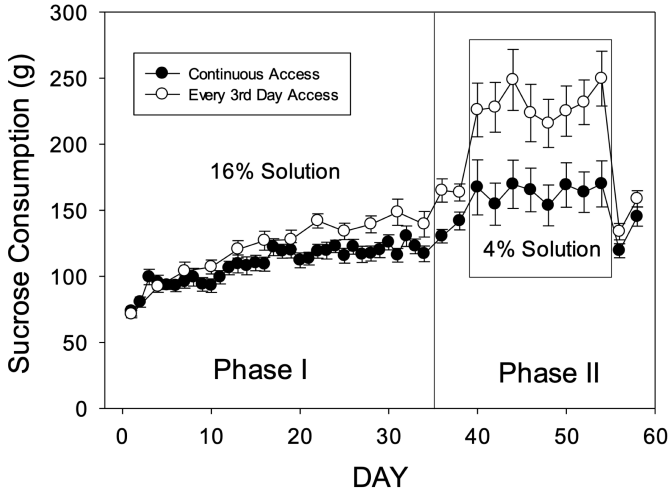
8-, and 16% sucrose (Eikelboom et al., unpublished). With a relatively weak 1% sucrose solution the IAE was not evident, possibly because 1% sucrose does not provide enough value or reward in terms of taste. For higher concentrations, differences in total volume of sugar solution consumed are less evident. The lack of a reliable volumetric intake difference for higher concentrations of sucrose likely involves limits on sucrose calorie consumption, satiation, and the interaction between these processes.

With the typical version of the ICP (ED-E3D Phase I: E2D Phase II) but with a more concentrated 16% sucrose solution, only a relatively small Phase I sucrose intake difference was evident between ED and E3D groups. During the common E2D Phase II, following 2 exposures to 16% sucrose, rats were given a less concentrated 4% sucrose solution on 8 alternating days before 2 final exposures to the original 16% solution. The relatively small Phase I difference in volume consumed was maintained in Phase II during the first 2 exposures to 16% sucrose and immediately significantly increased during the 8 exposures to 4%. Finally, reintroduction of the 16% solution for the final 2 exposures reduced the consumption difference (Eikelboom et al., unpublished, see Figure 1.2 A).

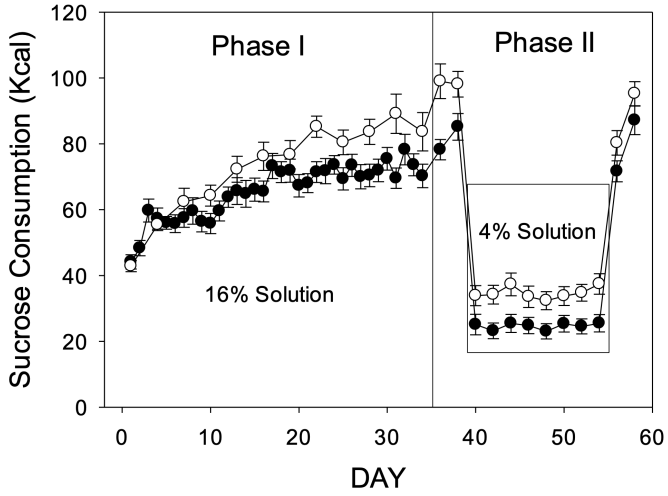
Figure 1.2

Intermittent-Continuous Protocol (ICP) with adult rats given 16% sucrose in Phase I. Mean (± SEM) solution intake in grams (A) and Kcal (B) for rats receiving solution every third day vs. every day in Phase I, and every second day in Phase II. For Phase II Days 40-54, rats received 4% solution (from Eikelboom et al., unpublished).

A)



B)



If total kilocalories consumed from sucrose are considered, the caloric intake difference between the ED and E3D group in Phase I was maintained in Phase II during the first two 16% exposures and remained stable for the 8 days when rats were given the less concentrated 4% sucrose solution (see Figure 1.2 B; Eikelboom et al., unpublished). Thus, the lack of a pronounced sucrose intake difference for higher sucrose concentrations is likely due to limits on fluid-volume and caloric intake.

Nutritive or caloric value is inferred mainly from taste by evolutionarily conserved receptors for sweet, sour, salty, bitter and umami. Eikelboom's lab has explored how taste impacts the access-induced consumption difference by the addition of quinine, a bitter, to varying concentrations of sucrose solutions. Work with sucrose-quinine mixtures shows that rats adjust their intake of sucrose solutions based on the taste of the solution and its availability (Valyear, 2014). Quinine adulteration (the addition of a bitter taste) used to degrade the taste of an 8% sucrose solution and consequently reduce intake, permitted the emergence of a large access induced sucrose intake difference that is normally only evident with 4% sucrose solutions (Valyear, 2014).

In the ICP, when rats are shifted to Phase II, the intermittent group has typically continued to consume stable levels of sucrose. In contrast, the continuous group has typically increased their sucrose intake but continued to consume less sucrose than the intermittent group (Eikelboom & Hewitt, 2016). Noted earlier, this differentiated sucrose consumption was evident after 50 days of Phase II, which clearly demonstrates that the access induced sweet solution

intake difference can be very persistent (Celejewski, 2011). Further exploring this effect, we tested the impact of longer-term ED or E3D access to sucrose followed by then reversing the access conditions for half of the animals (Senthinathan, 2012). Rats provided with continuous access to 4% sucrose for 40 days and then shifted to E3D access rapidly escalated their intake of sucrose after the switch and consumed a similar amount of sucrose solution as age-matched sugar naïve rats started on E3D access. Animals provided with 14 intermittent E3D exposures to the sucrose solution prior and then shifted to continuous access only very gradually reduced their sugar intake after the switch, eventually (after about 40 days) beginning to show consumption levels more typical for rats with continuous access on the final days. The period of intermittent E3D access to sucrose had sustained effects on sucrose consumption, resulting in a prolonged increase of sucrose intake (Senthinathan, 2012).

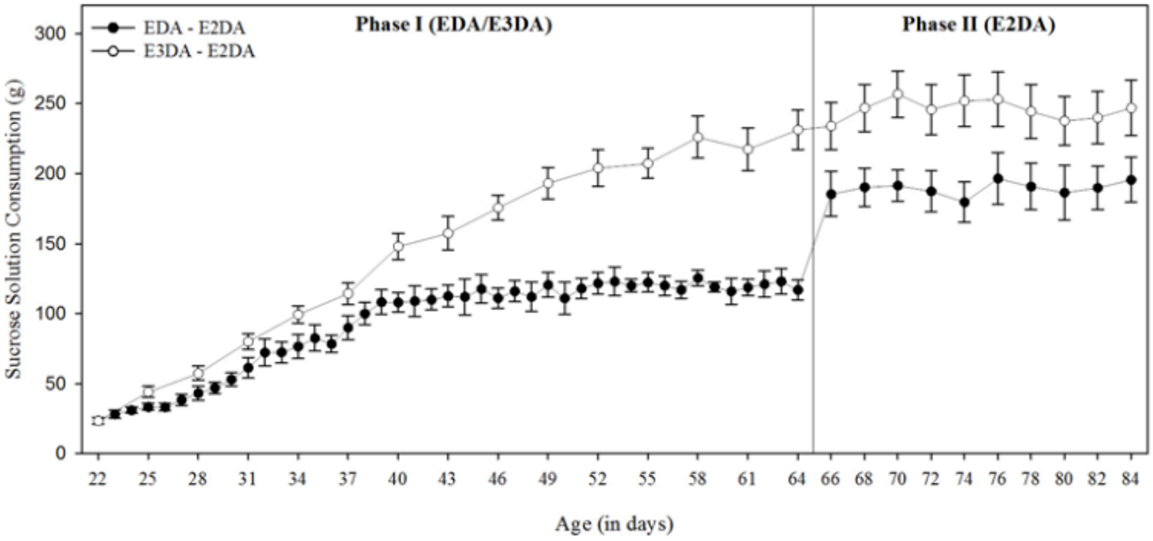
Only 2 experiments have investigated the relationship between age and access-induced sucrose consumption with the ICP. To test how age impacts the access-induced sucrose intake difference, we gave older aged rats (retired breeders weighing ~500 g at the beginning of the experiment) ED or E4D access to 4% sucrose solution for 17 days, or 5 intermittent exposures, respectively (Senthinathan, 2012). Following their first exposure to sucrose, the continuous group reduced their daily intake of sucrose from 183 g and stably consumed approximately 145 g per day while the intermittent group increased their consumption of sucrose to about 280 g whenever it was available (Senthinathan, 2012). Like work with younger adult rats, for older aged rats provided with a 4%

sucrose solution, the access-induced sucrose intake difference was both large and emerged very quickly. Given that a large consumption difference emerged quickly in Phase I, we had no reason to expect that the difference would not be maintained in Phase II. As such, and because of other experimental considerations with these older aged rats, we did not test these rats in a common Phase II.

The second attempt to look at development and access induced sucrose consumption involved very young rats. Pups (22 days old) were given E3D or ED access to a 4% sucrose solution for 15 intermittent exposures or 43 days continuous access (Phase I). As adults (aged 64-65 days) all rats were switched to an alternate day access schedule for Phase II (i.e. common E2D access Phase II). The typical sucrose consumption E3D/ED difference was not found in the pups, but it gradually emerged when the pups reached adolescence. Both ED and E3D groups escalated their intake of sucrose equally until approximately 39 days of age (see Figure 1.3). At this age, intake of sucrose solution by the continuous group began to stabilize at about 110 g per day while the E3D group continued to increase their intake of sucrose until about 58 days of age when their intake became stable at about 220 g of solution per day. In Phase II, at 64-65 days of age, both groups were shifted to E2D access and the differentiated sucrose consumption persisted for the 20 days of Phase II.

Figure 1.3

Intermittent-Continuous Protocol (ICP) with rats given 4% sucrose in Phase I. Mean (± SEM) solution intake (g) for rats receiving solution every third day vs. every day for 64 days in Phase I. Both groups had every second day access to 4% for 20 days in Phase II (from Senthinathan, 2012).



Similar to previous work with adults, these studies with older aged rats and with pups failed to show any impact on body weight (Senthinathan, 2012). Why the IAE is less evident among pups but eventually emerges across the adolescent period needs to be explored further.

Intermittent Access Protocols: Interim Summary

I have highlighted 3 intermittent access protocols to describe how availability impacts the consumption of natural rewards. In addition to testing

consumption, the 12-12-h and M-W-F protocols have been used to explore behavioural changes that co-occur with the access-induced increase or change in reward intake. Repeated intermittent daily access to sugar and chow (the 12 h-12-h protocol) resulted in a binge of sucrose drinking by rats during the first hour of daily access (Colantuoni et al., 2001) and an overall increase of sucrose consumption during the 12-h access period, comparable to 12 h intake by rats with 24-h access to the sugar solution. Similarly, the M-W-F protocol has been used to show that after few weeks of intermittent 2-h M-W-F access to shortening, rats escalated their intake of fat and began to consume as much of the palatable food during the limited access period as a group or rats that had 24 h or continuous access to shortening and chow (Corwin & Wojnicki, 2006; Corwin et al., 2011). Rats given daily 2-h access to shortening maintained stable consumption (~1/3 of the amount consumed by rats with intermittent access). The reason rats with 2 h of daily access to the fat do not show increased intake over time, and rats with 2 h of access on M-W-F show the effect, is likely related to circadian influence on feeding. There may be important differences in how daily and longer-term consumption is regulated and impacted by intermittent access.

With the ICP, we typically measure daily consumption. Comparing daily sucrose intake between rats with intermittent vs. continuous access to a 4% solution we typically find a large, almost 2:1 sucrose consumption difference respectively. Typically, with a sweeter solution (8% or higher), 24 h consumption differences are minimal, but can be demonstrated to exist with a shift to a 4%

solution in phase II. This sucrose consumption difference was evident during the first hour of access and maintained during each of the following 6 h that were examined. With an 8% sucrose solution Valyear (2014) found that the largest sucrose intake difference occurred during the first hour of access but subsequent hourly intake between E3D and ED groups was comparable (Valyear, 2014). Notably, this result suggests that some differences may be missed with low-resolution studies aimed at investigating consumption behaviour during a given time period. Higher-resolution studies are currently being done with the ICP to explore how intermittent access to sucrose impacts the temporal profile of sugar consumption (Celejewski, unpublished).

Intermittent Access Protocols: Investigating Neurochemical, Cellular, and Molecular Changes

I used the ICP to test rats and found differentiated sucrose consumption in experiments that are described in this document. I was interested in discovering the brain areas and mechanisms that underlie this differentiated sucrose consumption. The neural control of feeding is complex. Endogenous neurotransmitters including dopamine (DA), acetylcholine (ACh), serotonin, glutamate, GABA, opioids, and orexin have all been implicated in the regulation of feeding (Avena et al., 2008). Hoebel's lab was among the first to explore the commonalities that exist in the neural regulation of food and drink consumption, and drug taking (Avena & Hoebel, 2003). Because common brain areas are activated by natural rewards and drug consumption, Hoebel and colleagues tested if neuromodulators known to be influenced by repeated drug taking,

including dopaminergic, cholinergic, and opioid neurotransmission, are similarly influenced by repeated periods of intermittent access to sucrose with the 12 h-12h protocol (see review by Avena et al., 2008). Overall, rats with intermittent access to sucrose develop neural patterns that are typically associated with repeated drug consumption. For example, one study demonstrated rats with intermittent 12-h sucrose access develop transcriptional changes in several receptor types similar to those identified in morphine-dependent rats. Both morphine-dependent rats and 12-h sucrose rats had reduced D_2 mRNA, opioid mRNA, and increased D_3 mRNA in the forebrain. If repeated drug taking and intermittent sucrose intake induce similar transcriptional changes, this might suggest the neural mechanisms that underlie sustained excessive drug use over time (a hallmark of addiction) might also be involved in excessive sugar intake. Accumulation of the transcription factor ΔFosB in reward-related neural circuitry might be one of the mechanisms involved in development of sustained excessive consumption (Nestler et al., 2001), but several neural processes are likely involved.

To explore how chronic intermittent access to sugar impacts neural structures at the molecular level, Shariff and colleagues (2016) provided *ad lib* fed rats with 24 h intermittent access to a 5% sucrose solution on M-W-F for 4 or 9 weeks. A control group of rats were given continuous access to the sugar solution for 4 weeks; for the 9-week exposure, there was no continuous control group (minimal behavioural data was provided). After consumption stabilized rats were administered Varenicline (.03, 1, 2 mg), a partial agonist of the nicotinic

acetylcholine receptor (nAChR) (Shariff et al., 2016). Subsequently, rats were given a two-bottle choice (one bottle with water, and the other with 5% sucrose) test to explore how administration of the drug impacted sucrose consumption patterns. For the short exposure group only the 2 mg dose of Varenicline reduced sucrose consumption, and only for rats on the intermittent schedule. This result demonstrates that sugar consumption associated with intermittent access uniquely contributed to some neural modification upon which Varenicline had an effect. For the long-exposure condition, which only tested animals following the intermittent 24 h M-W-F schedule, both the 1 and 2 mg doses were effective in reducing sucrose consumption as measured at 30 min. Continuing to explore how nAChRs modulate intermittent access-induced changes in sugar consumption using the same two bottle choice test, Shariff and colleagues also found that Mecamylamine, a non-competitive, non-selective nAChR antagonist, and cytisine, a β2-selective nAChR agonist had a similar effect reducing the intermittent induced change in sucrose intake (Shariff et al., 2016). To explain the reduced sugar consumption with intermittent access by administration of partial agonists, agonists, but also antagonists of the nicotinic receptor, Shariff and colleagues suggest nAChR desensitization may play a role in sucrose consumption.

Following short (4 week) and long (9 week) sucrose exposure, using autoradiography to explore how sucrose consumption impacts the nucleus accumbens (NAc), a key reward structure in the brains reward system, Shariff and colleagues (2016) found increased alpha 4 beta 2 and decreased alpha 6

beta 2 receptors (both are nAChR subtypes) expression among rats maintained on an intermittent schedule compared to sugar-naïve control rats. Both nAChR subtypes are known to modulate dopaminergic reward-related activity in the NAc and ventral tegmental area (VTA) (Grady et al., 2010). These neural modifications in the NAc might underlie some of the increased consumption of sugar that is commonly induced by intermittent-access schedules (Avena et al., 2008; Corwin et al., 2011; Eikelboom & Hewitt, 2016; Rhen & Boakes, 2019).

Subsequently, Klenowski and colleagues (2016) used Golgi-Cox staining to assess whether intermittent access to sucrose facilitates changes in neuronal morphology including soma volume, total dendritic length, mean tree length, number of nodes and endings, and spine density in the NAc following short- and longer-term binge like sucrose consumption with the same two bottle choice protocol as described above. For the NAc core, cellular morphology remained relatively intact after short- and long-term intermittent access. Analysis of morphometric parameters following intermittent short- and long-term exposure to sucrose failed to reveal any significant differences in the NAc core and shell compared to age-matched water controls. In the NAc shell, the long exposure intermittent group had significantly decreased dendritic length, decreased dendritic complexity but increased mean spine density at distal branch orders. The implications of these morphological changes are not clear (Klenowski et al., 2016). Taken together, these studies by the Bartlett group demonstrate that access schedule and length of experiment are important when assessing how intermittent access-induced sugar consumption will impact reward-related

behaviour, neural chemistry, and neuronal morphology (Shariff et al., 2016; Klenowski et al., 2016).

The Immediate Early Gene c-Fos

With the ICP, intermittent access to sugar results in its increased intake in the longer-term (Senthinathan, 2012). Presumably the differential pattern of sucrose consumption and prolonged increase of sugar intake by rats that have had a period of intermittent access to sugar is supported by some change in the neural processing of sugar. The underlying differences in neural activation that support this robust difference in sucrose consumption behaviour remain unclear. Quantification of immediate early gene expression has been suggested as a viable way to explore neural activation in freely behaving animals (Dragunow & Faull, 1989). This procedure affords the comparison of neural activation associated with sugar consumption between rats that have been previously provided with continuous or intermittent access to sucrose.

The analysis of FOS expression has been compared to functional neuroimaging techniques that are purposed to measure brain activity by capturing some substrate of neural activity in real time or at a particular instance (Stark et al., 2006); immunohistochemical labeling and quantification of FOS protein provides excellent spatial albeit lesser temporal resolution than functional magnetic resonance imaging (fMRI). Neuroimaging techniques have been adapted to study small non-human animals however subjects are usually anesthetized to prevent movement during imaging procedures thus brain-imaging

work with non-human animals has focused on exploring neural structures and structural connectivity.

Exploratory whole-brain mapping studies can identify the specific neural systems that are associated with a given behaviour (Osten & Margrie, 2013; Perit et al., 2012). The following section describes c-fos and the use of Fos-expression for mapping neural excitation. Subsequently, studies that have used Fos-immunochemistry to explore how the consumption of food is processed by the brain are described.

Gene expression is the process by which genetic information is used to synthesize functional gene products that ultimately determine an organism's phenotype. This process involves transcription or copying of deoxyribonucleic acid (DNA) to messenger RNA (mRNA) which occurs in the nucleus of a cell and subsequent translation or protein synthesis on a ribosome in the cell`s cytoplasm. Thus, mRNA and protein are products of gene expression. Immediate early genes (IEGs) are genes that are transcribed and translated rapidly in response to cellular stimulation. They are activated in many processes such as learning, development, and growth (Dragunow, 1996; Dragunow & Faull, 1989; Herrera & Robertson, 1996; Pérez-Cadahía et al., 2011). Activation of IEGs contributes to long-term changes in neural plasticity, the nerve cell's ability to show acute or long-lasting phenotypic changes in response to external stimuli or cellular processes (Herrera & Robertson, 1996). While about 40 IEGs have been

identified, the immediate early gene *c-fos* is among the most widely studied and best characterized (for review see Herrera & Robertson, 1996).

The IEG *c-fos* is a highly conserved proto-oncogene[3] found in the cellular DNA of organisms throughout the animal kingdom. It is involved in a variety of cellular functions including proliferation, differentiation, and survival. A variety of cell types found throughout the body and nervous system express *c-fos*, many of which express high basal levels of c-fos mRNA and FOS (the protein product of *c-fos*). Importantly, neurons express low basal levels of the c-fos mRNA and FOS, however for neural cells the expression of *c-fos* is inducible[4] (Ahmad & Ismail, 2002). In other words, various behaviours and stimuli can activate the *c-fos* gene and cause the release of FOS protein.

Sagar and colleagues appeared to be the first to suggest that the activation of *c-fos* could be used as a high-resolution metabolic marker of neural activity in the central nervous system (Sagar et al., 1988). Dragunow and colleagues then demonstrated the use of FOS as a metabolic marker of neural activity by quantifying its expression after eliciting seizures in rats and mapping neural pathways associated with the spread of seizure activity (Dragunow & Robertson, 1987; Dragunow et al., 1988). Mugnaini and colleagues (1989)

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[3] Proto-oncogenes are genes found in cellular nuclei, these genes code for proteins which regulate cell growth. Consequently, a change in sequencing of the *c-fos* gene can give rise to oncogenes that interfere with normal cellular functioning and promote the development of tumor cells.

[4] Gene induction refers to the process by which stimuli increase gene expression.

showed that neural excitation by administration of Metrazol, a circulatory and respiratory stimulant, induces Fos-like immunoreactivity in neuronal nuclei, however no Fos-like immunoreactivity was observed within glial or endothelial cells. Light, auditory stimuli, pain, and other sensory stimuli, motor behaviours and stimulation of the motor cortex, as well as various drugs and toxins have been shown to induce IEG expression in neural cells (Sharp et al., 1993). In neural cells, following depolarization, the IEG *c-fos* is rapidly transcribed to c-fos mRNA and translated to FOS; following acute stimulation of a nerve cell, the expression of c-fos mRNA peaks within 30 minutes and subsequently the expression of the FOS peaks within about 60-90 minutes (Herrera & Robertson, 1996). Although the precise role of *c-fos* in specific neuroendocrine systems is unclear, in neurons, the expression of c-fos mRNA or FOS protein is indicative of recent neural activation and thus can be used as a biological marker of cellular activity (Dragunow & Faull, 1989).

Immunohistochemical and in situ hybridization techniques can be used to localize FOS proteins and mRNA respectively among distinct cellular populations, providing a powerful tool for the assessment of neural activation in brain mapping studies. Given that FOS-immunoreactivity (FOS-IR) can be identified in anatomically discrete brain regions, and that every distinguishable brain structure can be studied, quantification of FOS-IR permits a rigorous investigation of the neuroanatomical distribution of activity associated with a given behaviour.

The expression of FOS protein occurs a predictable time after activation; this delay affords the mapping of neural activity that is associated with recent behaviour. More clearly, following stimulation of a neuron the expression of FOS protein is delayed for about 30-45 minutes thus allowing for experimenter handling and environmental change before euthanasia that will not result in stress induced or non-specific FOS expression. This delayed activation makes the protein product of the immediate early gene *c-fos* (i.e. FOS) an ideal anatomical marker of neuronal activation.

I used immunochemistry and bright-field microscopy to visualize and quantify the expression of the immediate early gene *c-fos* following the consumption of the sweet solution (Chapter 4). Following a period of continuous or intermittent access to sucrose, identifying neural structures associated with the consumption of sucrose and comparing findings between these groups could provide insight into the mechanisms by which an elevated pattern of sucrose consumption is maintained.

To stain tissue and visualize FOS expressed by the gene *c-fos* some studies have uses antibodies that react with FOS as well as other FOS-related nuclear antigens. For these studies the visualized protein expression may not be specific *c-fos* thus it is referred as FOS-like. Similarly, some studies describe c-FOS-like immunoreactivity, this is because the antigens used in these studies react with all members of the Fos family (e.g. delta FosB, cjun, etc). For the FOS experiment in this dissertation I used c-fos primary antibody, which is specific to FOS expressed by the IEG *c-fos.*

Complex Network Analysis

FOS-IR datasets are usually analyzed at the micro level (i.e. discrete analysis) (Lattemann et al., 2011; Haino et al., 2010; Koh et al., 2003; Pecoraro & Dallman, 2005; Pomonis et al, 2000; Yamamoto & Sawa, 2000; Yamamoto, 2003). Analysis of the activation among discrete brain areas via FOS-IR can be used to determine if a single brain area shows increased or decreased activity following a given treatment (akin to a micro view of the data) by comparing neural activation among experimental and control animals. However, discrete analysis of FOS-IR does not provide any information about how the system is functioning as a whole (akin to a macro view of the data). Differences that may be evident at the micro level may not be evident at the macro level of analysis, and vice versa. Complex behaviours are not just supported by individual brain structures, but also by highly organized networks of neural networks. This connectivity of brain structures can be described as structural or functional. Brain areas are structurally connected if they are innervated or joined by tracts. Functional connectivity in neural networks refers to correlated brain activity among anatomically discrete brain areas. It is important to consider functional connectivity and properties of functional neural networks among discrete brain structures to gain a better understanding about how complex behaviours such as feeding, and drinking are regulated. Complex network analysis is a fairly novel technique in the neural sciences that can be used to uncover functional neural networks and analyze properties of these neural networks (Rubinov & Sporns, 2010).

Individually, both the micro and macro level of analysis are useful for exploring FOS-IR datasets but when applied together they provide a richer or more complex view of the data. I performed Fos-immunochemistry experiments (Chapter 4 Exp. 1), and subsequently explored the neural network properties of these datasets (Chapter 4 Exp. 2) in MatLab. For a complete review of neural network parameters or the complex network analysis technique see Rubinov and Sporns (2010). A brief description of these network parameters is provided in Chapter 4.

Consumption of Sugar and Fos Expression

Consumption of carbohydrates, fats, and other macro-nutrients induces Fos-IR in gustatory and reward-related brain areas including the nucleus of the tractus solitarus (NTS; the primary taste nucleus which receives orosensory information from the tongue via facial and glossopharyngeal nerves) and the ventral tegmental area (VTA; the main site for reward-related dopaminergic cell bodies that projects to the NAc and amygdala) (Norgren et al., 2006; Schwarz et al., 2010; Yamamoto & Sawa, 2000). Among studies that have explored the relationship between sugar consumption and Fos-IR, results have shown commonalities, as well as discordance, possibly due to the method of sucrose delivery, sucrose concentration used, developmental age of the rats, and previous experience with sucrose or other sweets (Dela Cruz, 2015; 2016; Lattemann et al., 2011; Haino et al., 2010; Koh et al., 2003; Pecoraro & Dallman, 2005; Pomonis et al, 2000; Yamamoto & Sawa, 2000; Yamamoto, 2003). In sucrose sham-drinking studies post-ingestive signals are reduced, thus Fos-IR is

attributed to orosensory effects (Norgren et al., 2006). The influence of taste is avoided with the sucrose gavage technique (intragastric infusion), which can highlight Fos-IR associated with post-ingestive effects of consumption because rats are not permitted to taste the solution (Yamamoto & Sawa, 2000). Fos IR-associated with both intraoral infusion of sucrose (Yamamoto & Sawa, 2000) and voluntary drinking (Dela Cruz, 2016), can be attributed to taste and post-ingestive effects. Fos-IR is an excellent tool for spatial mapping of single-cell activation associated with sucrose intake (Dela Cruz, 2016; Pecoraro & Dallman, 2005).

Anatomically discrete brain areas showing strong correlations in the pattern of FOS-IR induced by the consumption of sucrose suggests a functional relationship among disparate neural structures, supporting a distributed brain network mediating sugar intake. To explore simultaneous neural activation in forebrain meso-corticolimbic and nigrostriatal dopamine (DA) systems associated with the consumption of sweet solutions and fat, Dela Cruz and colleagues (2016) gave groups of rats access to 10 ml of a solution for 1 h. Each group was tested with specific solutions, including groups that were tested with water, a corn oil solution (fat), 8% fructose, and 8% glucose. Ninety minutes after initial exposure to the test solution, rats were anesthetized by intraperitonial injection (IP) of sodium pentobarbital, perfused transcardially, and tissue was prepared for FOS-immunolabelling. Quantifying and comparing FOS-IR in 8 discrete reward-related brain areas revealed that FOS-IR was increased following the consumption of sugar and fat; further analysis showed significant correlations in FOS-IR among forebrain areas including the amygdala, the NAc, and the medial

prefrontal cortex which the authors suggest is evidence for a distributed brain network mediating the intake of sugars and fats.

Chapter 1 Summary

Work with various intermittent access protocols shares the following commonality: when increased sucrose consumption was reported, this behaviour was observed in rats that had limited access to the rewarding substance, and typically compared to other rats that had more frequent availability (Corwin et al., 2011). In these models of increased sucrose consumption, the change, or increased sucrose consumption behaviour, may reflect learning. In other words, rats are learning to adjust their behaviour (i.e. sucrose consumption) during a period when availability of a resource is infrequent or uncertain.

We have long understood that environmental constraints such as infrequent or limited availability can strongly influence how rats consume an item (Sinclair & Senter, 1967). Why do rats increase their intake of items that are only available intermittently? In other words, why would this behaviour pattern have evolved, or how does it provide some competitive advantage? Rats given intermittent access to ethanol, as well as various non-drug solutions, increased their intake compared to rats with more frequent access (Wayner & Fraley, 1972; Wayner et al., 1972, Wise, 1973, Avena et al., 2008). This change in consumption behaviour (i.e. increased intake) seemed to be due to the limited availability and must have been supported by adaptation in some neural system. From an evolutionary standpoint, a system that is flexible, or able to adapt to the availability of nutriments and influence behaviour, could increase an organism's

fitness by impacting its consumption of vital nutrients that are not regularly available.

The way availability influences consumption is controlled by a neural system for regulating motivated behaviour, which is highly conserved among human and non-human animals (Grill & Norgren 1978; Steiner et al. 2001). The relationship between availability, patterns of consumption, and related neural processes is complex because it involves reciprocal relationships. For example, the availability of an item, or how often is encountered, will influence how it is processed by the brain. This processing can influence how the item is consumed (e.g. increased consumption vs. normal consumption). Similarly, the way an item is consumed (i.e. increased consumption vs. normal consumption) will uniquely engage the brain's reward system, and this neural activity will influence how the brain processes the item the next time it becomes available, ultimately guiding behaviour.

The relationship between availability, consumption, and related neural processes is difficult to disentangle. I used a rat model of increased sucrose consumption behaviour (the ICP) to explore how availability affects the intake of sugar solutions and to explore neural activity associated with increased sucrose consumption. In my MSc work I had used the same protocol and obtained some results with very young rats that contrasted what we reliably find with adult rats (Senthinathan, 2012); in my dissertation I focused on exploring this finding. By testing how availability impacts sugar consumption across age-development I hoped to gain some insight into how availability and restriction can influence the

consumption of sugary food, drink, and other rewards. My goal in my dissertation was to contribute to our understanding of how availability or restriction influences consumption behaviour. I was particularly interested in addressing the following question: When considering how availability or restriction influences consumption behaviour, does age or developmental stage matter?

Chapter 2: Sucrose Intake in Pups, Adolescents, and Adults

The ICP results with younger rats showed that the differentiated sucrose intake develops slowly (Senthinathan, 2012), whereas work with adult rats demonstrated the differentiated sucrose intake behaviour develops rapidly (Eikelboom & Hewitt, 2016). I had found pups did not show the access-induced (continuous vs. intermittent) sucrose solution intake difference, and the difference develops over adolescence (Senthinathan, 2012). Other work has shown sucrose preference changes developmentally, and supports adolescence is associated with a gradual transition from pup behaviour, to that more typical of adults. For example, younger rats choose to consume sweeter solutions than adult rats, and gradually transition to the adult pattern across the adolescent period (Bertino & Wehmer, 1981). Among pups, does the inclination for sweets, or some other age-related phenomena, prevent or protect them from developing the access-induced increased sucrose consumption?

Exposing younger (adolescent) rats to sucrose can have long-term influence on consumption related behaviours when compared to rats first exposed as adults (Vendruscolo et al., 2010). Younger rats (30-46 days of age) given continuous access to 5% sucrose for 17 days showed reduced motivation for a sweet non-caloric (saccharin), and non-sweet caloric (maltodextrin) solution in adulthood compared to sucrose naïve rats, and this result was specific to younger rats because a parallel procedure in adults produced a much less pronounce change. Other work had shown male rats given diets containing 0, 12, or 48% sucrose from 16-30 days of age (as pups) and then access to all three

diets until adulthood at 63 days did not present sucrose preference differences, suggesting the different sucrose experience as pups had no longer-term consequence (Wurtman & Wurtman, 1979), so the age-effect reported by Vendruscolo and colleagues (2010) might be due to the sucrose access the rats had as adolescents. The adolescent brain may be particularly sensitive because reward and motivation related areas in the brain undergo significant development and reorganization during this period (Zoratto et al., 2018), which might make adolescents more sensitive to developing longer-term behavioural changes (Spear, 2000; Simon & Moghaddam, 2015).

Experiment 1

I was interested in comparing sucrose consumption between pups, adolescents, and adults, including initial sucrose consumption, limited vs. continuous exposure to sucrose at each age (is the influence limited vs. continuous access similar at each age period), as well as longer-term consumption patterns in groups that had continuous access to sucrose from the beginning of each age period. To this end, I used a sequential design with three Age groups to test and compare rats as pups, adolescents and adults starting at 22, 39, and 56 days of age, respectively.

Age groups were equally split into three access conditions so that overall the first day consumption within age would not differ across groups (Table 1). Rats in the ED condition received sucrose continuously, after their first day of sucrose rats in the 4D condition received their second day of solution after a gap of two days, so on Day 4, and their third access 16 days later on Day 20, while

rats in the 20D condition received their second day of access after a gap of 19 days, on Day 20. This design permitted us to explore across these ages, the effects of a short 2-day gap and a longer 19-day gap on sucrose consumption in two major analysis that were then broken down further.

Table 2.1

*Experimental design showing days (in age) when rats received sucrose. In ED groups each (*) between days (e.g. 22 * * 25) represents 1 day with sucrose in every day (ED) groups. For each age, column shade (white, grey, black) parallels symbol color in figures in this chapter.*

Adults	**ED**	56 * * 59 * * * * * * * * * * * * * * * * 76
	4D	56 59 76
	20D	56 76
Adolescents	**ED**	39 * * 42 * * * * * * * * * * * * * 56 * * 59 * * * * * * * * * * * * * * * * 76
	4D	39 42 59
	20D	39 59
Pups	**ED**	22 * * 25 * * * * * * * * * * * * * 39 * * 42 * 76
	4D	22 25 42
	20D	22 42

This sequential design could address whether adolescence (or another age period) is particularly sensitive to limited, or continuous sucrose exposure. Continuous sucrose availability earlier in development desensitizes rats to sweets compared to similar experience at an older age (Vendruscolo et al., 2010), so we might expect rats given continuous access to sucrose as pups and adolescents would consume less sucrose in adulthood compared to animals that did not have the early experience. This experiment had several objectives including testing for age-mediated differences in volitional consumption of 4%

sucrose at the three developmental stages, exploring how the influence of restricted or intermittent access to 4% sucrose changes across the three developmental stages, and determining whether the age of onset of chronic sucrose availability impacts sucrose intake levels later in life.

Rat size limits the amount of sucrose solution that can be consumed. There are different ways to compare consumption by rats of varying size and age. I compared intake per 100 g of body weight to adjust for the various sized rats (Wilmouth & Spear, 2009). An alternative to this strategy to equalize and compare rats of varying size, we could have considered volume intake as a function of the total body-surface area of the rat (Nair & Jacob, 2016). There was no experimental consideration that suggested this analysis would be important, so I did not measure rat body-size in any experiment.

Methods

All procedures in the experiments in this dissertation were approved by the Wilfrid Laurier Animal Care Committee in accordance with the guidelines and policies from the Canadian council on Animal Care (Protocols R10001, R14005, R18006).

Caloric and nutrient requirement varies with age, sex, species, and rat strain. In this dissertation all experiments were carried out using male Sprague-Dawley rats as a practical way of reducing some of this complexity.

Subjects

Seventy-two Male Sprague-Dawley rats aged 21-22 days at arrival were ordered form Charles River Canada, St. Constant, Quebec. Rats were

individually housed in plastic shoebox cages (20 X 24 X 45 cm) maintained on a 12:12 light/dark cycle in a colony room having a room temperature of 21 ± 2°C.

Materials

Four percent sucrose solution was prepared using tap water and commercially available pure cane sugar, mixed on a weight/weight basis (4% solution – 4g of sugar for every 100g of solution) in 10L Nalgene jugs. The solution was prepared either one day prior to sucrose solution access or the day of sucrose solution access and made available to the rats at room temperature in glass bottles with metal drinking spouts.

Procedures

Water and rat chow (Harlan Tek-Lab 8640, 3.11kcal/g) were available continuously. Cage bedding (hard wood chips) in the cages was changed twice a week or more to maintain dry shoebox cages. Water bottles were changed every 7 days and sucrose bottles were changed daily. To reduce the likelihood of sucrose spillage, sucrose bottles were always placed at the location designed for water bottles, and water bottles were placed on the other side of the cage with food pellets in between. Following these procedures, spillage of sucrose solution is typically <1 g per day (Eikelboom & Hewitt, 2016). Daily sucrose intake was measure whenever rats received the solution. Sucrose bottles were weighed before and after each daily period rats had sucrose, and daily sucrose intake reported is always the difference in grams between these two consecutive measurements. Daily access was actually about 23h/day because animals did not have access to sucrose, water, or food, while measurements and other

procedures (changing cages and water bottles, topping up food, etc.) were completed. These daily procedures were completed during the light cycle at approximately the same time of day. Body-weights of all animals were measured following common sucrose access days and the final experimental day (Days 22, 25, 39, 42, 56, 59, and 76).

Rats were randomly assigned to one of 3 Age groups (n = 24) as follows: Pups, Adolescents, or Adults (Spear & Brake, 1983; Spear, 2000). These groups represent when rats were initially given 4% sucrose, which was at 22, 39, or 56 days of age, respectively (i.e. Pup groups were initially given sucrose at 22 days of age. When each age group had their first day with sucrose (Day 1), Pups, Adolescents, and Adults were pseudo-randomly assigned to one of three Access conditions (ED, 4D, 20D) balanced by sucrose intake on Day 1 to ensure initial sucrose intake between Access conditions for each group were equal. ED groups were given daily access to sucrose. D4 groups were given access to sucrose on Day 1, Day 4, and Day 20 (one day with sucrose, followed by a 2-day gap without sucrose, another day with sucrose, a subsequent 16-day gap, and one final day with sucrose e.g. the Pup 4D rats were given sugar at 22, 25, and 42 days of age). D20 groups were given access to sucrose only on Day 1 and Day 20. Thus, each Age group (n = 24) was split into 3 Access conditions (ED, D4, and D20 with n = 8 per condition for each age group). Table 1 shows the days rats had access to 4% sucrose.

Data Analysis

This experiment involved comparing daily sucrose solution intake in rats of different ages and sizes. Size differences impact the amount of solution rats can consume in a day, so to adjust for group size differences, I converted raw sucrose volume intake (grams consumed) into consumption per 100 g of body-weight data, and used this adjusted data as the dependent measure (grams consumed per 100 g of body-weight) in most of the statistical analysis in this chapter. Table A1 in Appendix A shows the body-weight data, and below it, Figure A1 shows the unadjusted sucrose volume intake in grams on all sucrose days, and Figure A2 shows this sucrose consumption data, adjusted by body-weight.

Statistical analysis was completed with IBM SPSS version 25. This experiment involved multiple days of sucrose availability. Statistical analysis was mixed analysis of variance (ANOVA), comparing consumption between groups of rats on common sucrose days (i.e. days when two or more groups received sucrose). The results for repeated-measures factors were considered significant ($p < 0.05$) only if also significant when using the Greenhouse-Geisser correction for violation of sphericity. Analysis of weight gain paralleled sucrose analysis.

Initial analysis was 3 Age by 2 Access (ED, 4D) repeated measures ANOVA comparing adjusted sucrose intake on Day 1 and Day 4. The second analysis was 3 Age by 2 Access (ED, 20D) repeated measures ANOVA comparing adjusted sucrose intake on Day 1 and Day 20 (Appendix B shows supplementary analysis of Day 1 and Day 20 in a 3 Age by 3 Access (ED, 4D,

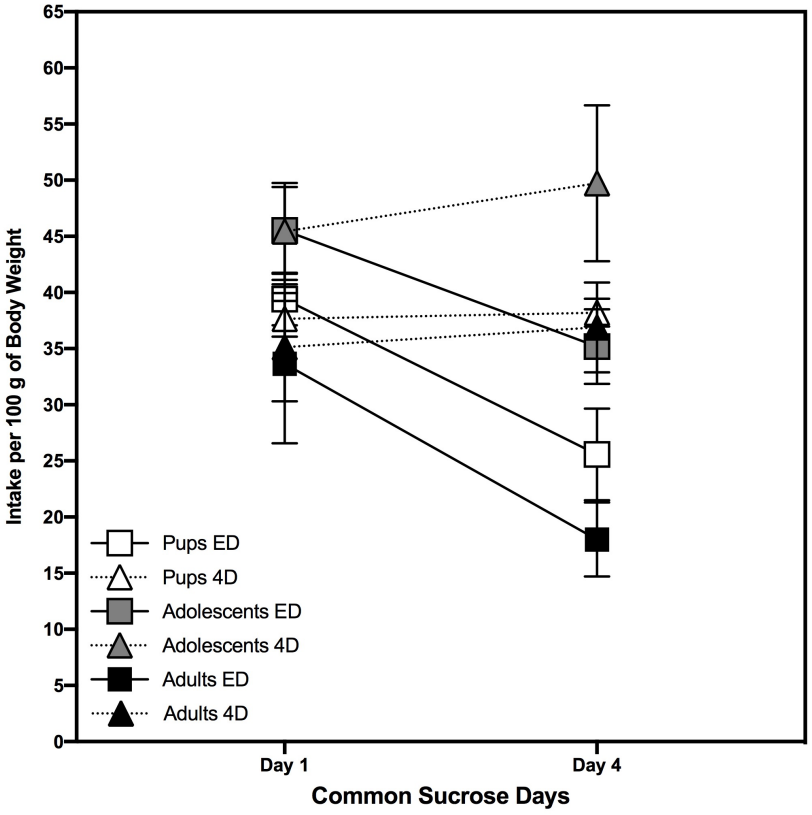
20D) repeated measures ANOVA comparing adjusted sucrose intake). Analysis of all groups explored body-weight data across the experiment. To test if age of onset of chronic sucrose availability impacted consumption levels in adulthood, sucrose intake levels in the ED groups was compared by repeated measures analysis of the final 14 experimental days in 2 7-day blocks.

Results and Discussion

The first major analysis compared the consumption of the ED and 4D groups on Day 1 and Day 4 in a mixed (Age by Access by Day) ANOVA. There was a significant Day by Access interaction, $F(1,42) = 9.18$, $p = .004$, $\eta_p^2 = .179$, reflecting how sucrose consumption decreased from Day 1 to Day 4 in the ED groups, while it increased in the 4D groups. Figure 2.1 shows the consumption of these six groups over the two days and it is evident that there were clear differences across the ages with the adolescent rats consuming more (consumption per 100 g) than pup and adult rats, reflected in a significant Age effect ($F(2,42) = 3.74$, $p = .032$, $\eta_p^2 = .151$). As there was were no significant interactions involving age it is evident that the age difference remained relatively stable across days and access conditions.

Figure 2.1

Mean (± SEM) sucrose intake per 100 g of body-weight on Day 1 and Day 4 by pup, adolescent, and adult every day (ED) and intermittent 4D groups.



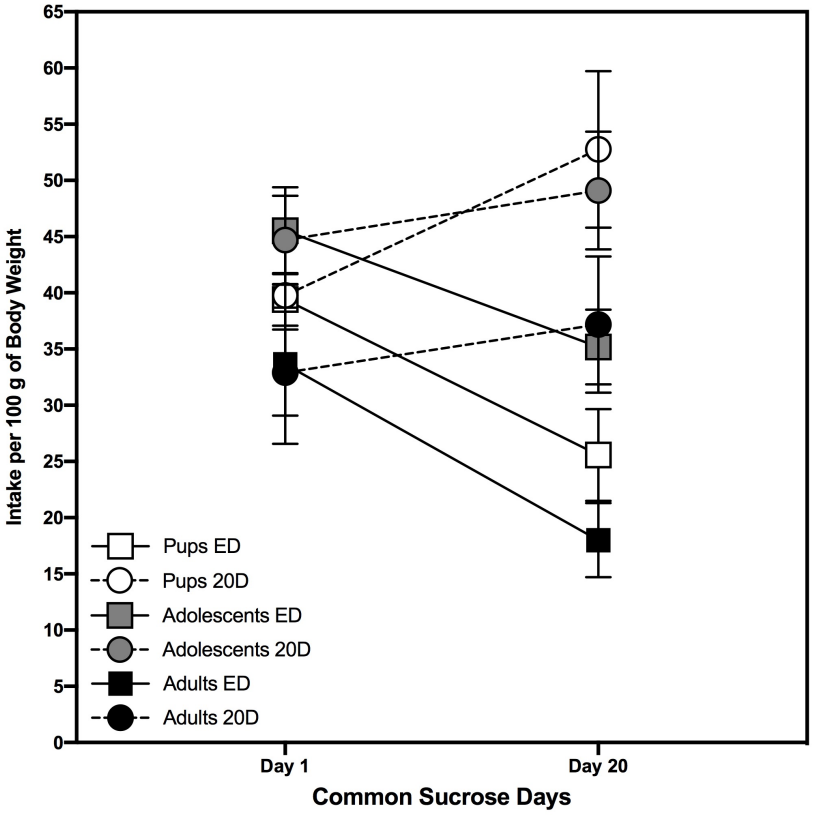
Subsequent single day ANOVAs of these 6 groups revealed, as expected, only an Age difference on Day 1 ($F(2,42) = 3.28$, $p = .048$, $\eta_p^2 = .135$) with adolescent rats consuming more than the other two ages. On Day 4 there were significant Age ($F(2,42) = 3.25$, $p = .049$, $\eta_p^2 = .134$) with adolescent rats consuming more than the other two ages, and Access ($F(1,42) = 4.14$, $p = .048$, $\eta_p^2 = .090$) main effects but no significant interaction suggesting that the three ages had similar access induced changes. At all ages the consumption was

lower in the continuous access conditions than in the intermittent access conditions.

A similar mixed ANOVA was carried out comparing Day 1 consumption to Day 20 consumption comparing ED rats to those in group 20D that received their second day of sucrose access on Day 20 for all three ages (see Figure 2.2). In this ANOVA there was a significant Age effect ($F(2,42) = 5.95$, $p = .005$, $\eta_p^2 = .221$) but no interactions involving Age suggesting that as in the previous Day 1, 4 comparisons adolescent rats consumed more in all situations than pup and adults. There was a significant Access effect ($F(1,42) = 9.54$, $p = .004$, $\eta_p^2 = .185$) and there was also a Day by Access interaction ($F(1,42) = 26.92$, $p < .001$, $\eta_p^2 = .391$). From Figure 2.2 it is evident that while consumption dropped from Day 1 in the ED groups it increased in the 20D groups.

Figure 2.2

Mean (± SEM) sucrose intake per 100 g of body-weight on Day 1 and Day 20 by pup, adolescent, and adult every day (ED) and intermittent 20D groups.



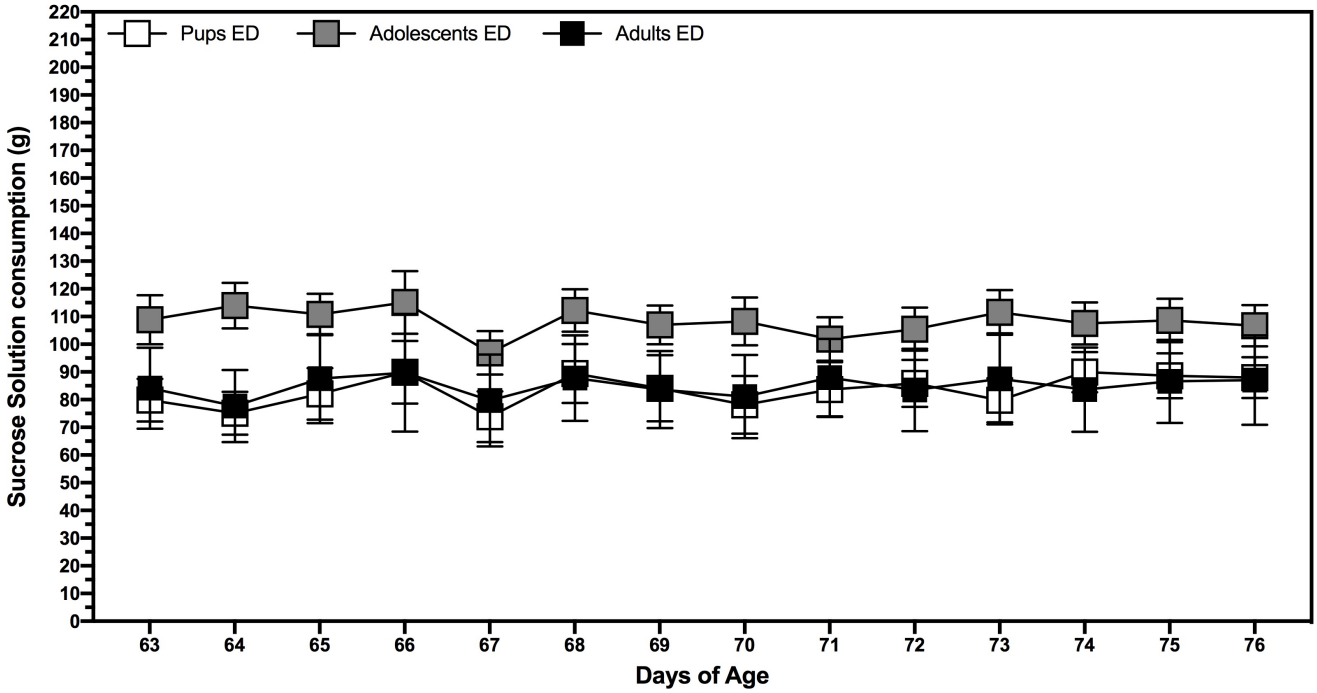
Subsequent single day ANOVAs of these 6 groups revealed, as expected, only an Age difference on Day 1 ($F(2,42) = 4.13$, $p = .023$, $\eta_p^2 = .164$) with adolescent rats consuming more than rats at the other ages. On Day 20 there were significant Age ($F(2,42) = 4.69$, $p = .015$, $\eta_p^2 = .182$) and Access ($F(1,42) = 24.03$, $p < .001$, $\eta_p^2 = .364$) main effects but no significant interaction suggesting that the three ages had similar access induced changes. At all ages the consumption was lower in the continuous access conditions than in the

intermittent access conditions with adolescent rats generally having a higher consumption than the other two groups. A parallel set of ANOVAs were run comparing Day 1 and Day 20 consumption for all ages and all three access conditions (ED, 4D, 20D), which revealed similar results, see Appendix B.

Pup, Adolescent, and Adult ED rats had continuous access to 4% sucrose beginning at 22, 39, or 56 days of age, respectively, until 76 days of age. Repeated measures analysis of body-weight data across the experiment at 22, 25, 39, 42, 56, 59, 76 days of age comparing all groups showed all rats gained body-weight across the experiment ($F(1,42) = 24.03$, $p < .001$, $\eta_p^2 = .364$) with no other significant effects, so Age and Condition did not influence weight gain. To assess whether earlier compared to later continuous sucrose availability influences consumption levels in adulthood, I compared sucrose consumption levels by the rats at 63-76 days of age (the last 14 days of the experiment). Since this comparison involves rats at the same age and weight, no size/weight adjustment was needed and volume of sucrose intake in grams was used as the dependent variable in this analysis. By the end of the experiment, sucrose intake levels by rats that had continuous access to sucrose from the pup, adolescent, and adult period, respectively, was similar (Figure 2.3).

Figure 2.3

Mean (± SEM) sucrose intake (g) in pup, adolescent, and adult every day (ED) groups on the final fourteen days.



Repeated measures analysis of the final 14 days of the experiment (rats were 63-76 days of age) in two equal blocks of seven days failed to reveal any differences between the three age groups, suggesting that the age at which rats were initially given continuous access to sucrose was not affecting intake levels at this point. Likewise, ANOVAs comparing intake between the Age groups (Pups, Adolescents, Adults) on the last day of each block (when rats were 69, and 76 days of age) failed to reveal any difference.

General Discussion

Initially, the adolescent groups consumed the most sucrose, and their intake adjusted for body-weight on Day 1 was greater than the adults. In comparison, the pup groups consumed a moderate amount, and their intake was not significantly different from the adolescent or adult groups. The elevated consumption in the adolescents compared to the other groups on Day 1 might be related to age-related differences in approach and avoidance behaviour associated with novelty seeking (Macri et al., 2002). The adolescent period has been associated with increased novelty seeking (Dahl, 2004; Kelley et al., 2004; Spear, 2000; Macri et al., 2002) and reduced novelty seeking is a common hallmark of normal ageing (Daffner et al., 1994).

The age-related differentiated sucrose intake observed on Day 1 was still evident on Day 4 and 20. This result suggests the novelty associated with consumption of sucrose on Day 1 was not the only important variable underlying the Age effect found on that day. The age-related difference in sucrose intake on Day 4 and 20 might be related age-mediated differences neural processing of palatable foods, drinks, and other rewards, as well as the influence of experience with sucrose during the different developmental periods.

The influence of intermittent vs. continuous access was not mediated by age. Intermittent groups maintained or increased their intake of sucrose per 100 g of body-weight compared to continuous groups in all cases and at all ages. This result was surprising given my previous work suggested the pattern (sucrose intake difference) might be different across the ages groups (Senthinathan, 2012). In subsequent experiments I focused on exploring this

further to clarify if the short- and longer-term influence of limited or restricted access is different in younger rats (pups).

The ED groups gradually decreased their intake of sucrose (adjusted for body-weight) across the experiment. These groups were initially given sucrose as pups, adolescents, or adults, and gained weight at a similar rate throughout the experiment. Comparing the three age groups in terms of uncorrected volume of sucrose consumed, the younger/smaller rats consumed less sucrose than the older/larger rats. The very small pups consumed a relatively small amount of 4% solution, and the relatively small adolescents (compared to the adults), consumed more solution than the pups, but less than the adults. As the pup and adolescent rats grew in size and weight, they increased their volume of solution intake. In the latter part of the adult period tested in the experiment (63-76 days in age) solution intake by the three ED groups was similar, so rats with continuous access to sucrose consumed similar levels of solution as adults, irrespective of when they first had sucrose. Therefore, the age (or developmental stage) at which rats were given chronic access to sucrose did not affect daily sucrose intake levels in adulthood. Other work has shown a period of continuous access to sucrose across part of the pup period and into adolescence has long-term influence on sweet-consumption behaviour, demonstrated by reduced motivation for sweets compared to adults following parallel experience (Vendruscolo et al., 2010). The reason I did not find a similar age-related difference might be because the procedures used by Vendruscolo and colleagues were more sensitive to behavioural differences.

It is known that sucrose preference changes developmentally, and younger rats choose to consume sweeter solutions than adult rats. Rats gradually transition to more adult-like sucrose behaviour across the adolescent period (Bertino & Wehmer, 1981). The neural changes responsible for this age-related decline in preference for more intense sweetness are not known. Just one or two short- or longer- gaps without sucrose can influence sucrose consumption patterns in rats. It may be important to consider how repeated periods of intermittent access to sweets early in development contributes to consumption behaviour in the short- and longer-term.

Chapter 3: ICP in Pups and Adults

The pup, adolescent, and adulthood periods in the rat are described in Chapter 1. Developmental work has suggested pups regulate food intake more tightly than older animals. For example, interventions that reliably increase (Swithers et al., 2004), or decrease (Dalton-Jez, 2006) feeding behaviour in older rats do not influence feeding behaviour in pups (*see Chapter 1: Focus on Pups*). Mirroring these findings, the ICP reliably differentiates sucrose intake between older intermittent and continuous groups, and this IAE is not evident in pups (Senthinathan, 2012). Contrastingly, in Chapter 2 with a different intermittent/continuous design I found the influence of availability on patterns of sucrose intake is similar across the three developmental periods. To better understand how availability influences sucrose intake in pups compared to older rats, here, I tested pups and adults separately in several experiments. I used the ICP in these experiments because I was particularly interested in longer-term change, which can be explored in Phase II of the ICP. Rather than the traditional terminology (IAE), I will be using the term “ICP Phase I effect” to describe possible IAEs in Phase I and “ICP Phase II effect” to describe possible IAEs in Phase II.

Feeding behaviour during the pup period might be uniquely regulated by developmental mechanisms that provide protection for the developing brain that is particularly vulnerable to disruption from malnutrition (Rosenzweig & Bennett, 1996; Spear, 2000). Such functionally adaptive mechanisms make sense from an

evolutionary perspective and might render pups resistant to developing maladaptive behavioural patterns related to feeding.

With the ICP, resistance can be considered in the short- (Phase I) and longer-term (Phase II). In my MSc work I gave pups intermittent vs. continuous access to 4% sucrose from 22 to 64 days of age (i.e. across pup and adolescent periods, and into adulthood). As pups, the groups showed resistance to the Phase I effect we typically find with adults given 4% sucrose, and the difference gradually emerged across the adolescent period (Senthinathan, 2012). It is possible that longer-term behavioural differences developed during the pup period even though the groups did not show a sucrose intake difference as pups. In support, work with the ICP in adults has shown a sucrose intake difference in Phase I is not critical for development of longer-term change (Eikelboom et al., unpublished). This work with is described in Chapter 1 under ("The Intermittent vs. Continuous Protocol" heading) and summarized below.

With adult rats given intermittent vs continuous access to 4% sucrose, the intake difference typically develops very quickly, presenting about a two-fold difference in daily sucrose intake between the groups in Phase I that is maintained in a Phase II with alternate-day access. With 16%, the groups consume similar amounts in Phase I and initially in Phase II. Following Phase I and a few days of alternate day access in Phase II with 16% sucrose, Eikelboom and colleagues gave the groups 4% sucrose instead of 16% on an alternate day basis, and surprisingly, the intermittent group consumed more than the continuous group on these sucrose days (Figure 1.2 A and B in Chapter 1). In

other words, the difference emerged on these days with 4% demonstrating that an underlying difference was induced by the pattern of availability in Phase I. Thus, with the ICP adult rats do not always show differences in Phase I, but these groups can still develop and demonstrate a longer-term pattern of differentiated sucrose consumption. Somehow this difference was prevented from presenting with 16% in both phases and was effectively unmasked by giving the groups 4% in Phase II. Satiety mechanisms might prevent differentiated consumption behaviour from presenting with higher sucrose concentrations.

In pups, to test if intermittent vs. continuous sucrose availability influences behaviour in the longer-term with the ICP, the groups must only experience the sucrose access difference (i.e. intermittent vs. continuous access, Phase I of the ICP) as pups. My earlier ICP work showed rats with intermittent vs. continuous consumed similar amounts of 4% sucrose from 22 to about 39 days of age (Senthinathan, 2012). Following the pup period, differentiated sucrose intake very gradually developed into about a two-fold difference from ~39-58 days of age. At 64 days of age these rats were shifted to a uniform alternate day schedule in a Phase II and the significant group difference was maintained for the ten Phase II sucrose days. The initial difference that emerged across the adolescent period might have been solely due to the intermittent vs. continuous access during adolescence. If these rats were shifted to a uniform alternate-day schedule (Phase II) at 39 days of age (at the end of the pup period), would a difference still gradually emerge? I used this Phase I: Phase II design to isolate the pup period and test if the ICP can induce longer-term changes in pups. With

this design sucrose intake differences in Phase II would clearly demonstrate that pups are not resistant to the longer-term behavioural changes associated with the ICP.

This chapter describes six parallel behavioural experiments using the ICP with adults (Experiments 1 and 3) and pups (Experiments 2 and 4-6) that were designed to clarify the relative vulnerability to access-induced changes in sucrose consumption behaviour in pups compared to adults. Each of these experiments had 2-phases and two groups (except Experiment 6). Rats were given every day (ED) or every third day (E3D) access to sucrose in Phase I for 16 days (6 intermittent exposures). In Experiments 1 (with adults) and 2 (with pups) rats were given 4% sucrose in Phase I. In all subsequent experiments the groups were given 16% sucrose in Phase I. In Phase II of all these experiments rats were given alternate-day access to 4% sucrose. The duration of Phase II ranged from 5-15 common sucrose days.

In the experiments involving pups, Phase I spanned the pup period (22-37 days of age). Coinciding with the end of the pup period and the beginning of adolescence, Phase II began at 39 days. A sucrose intake difference during adolescence would demonstrate pups are vulnerable to the influence of availability on feeding behaviour. Alternatively, if pups are invulnerable to the development and later expression of increased sucrose consumption then this procedure should not result in any sucrose intake difference at any point in these experiments with pups. Results from Experiments 4 were surprising and were

mirrored in a replication study (Experiment 5). In Experiment 6, *the gap experiment*, we followed up on these findings.

General Materials and Methods

Aside from details noted below general materials and methods used for these experiments are identical to those described in Chapter 2.

Procedures. Adults were given seven days to acclimate to individual housing conditions and the new colony room. Pups were given only one day to acclimate to maximize the number of intermittent/continuous sucrose days during the pup period. Each experiment had 2 phases. In Phase I rats were matched on Day 1 sucrose intake and pseudo-randomly assigned to two equal groups balanced by this intake. The ED groups had continuous access to sucrose for 16 days while the E3D groups had intermittent access to the same solution every third day (Day 1, 4, 7, 10, 13, and 16). In Phase II, and beginning on Day 18, both groups were given alternate-day access to 4% sucrose for 5-15 common sucrose days.

Statistics. All experiments in this chapter had two phases with multiple common days of sucrose availability. Phases were analyzed separately, and primary analysis was between-group repeated measures analysis of variance (ANOVA) for common sucrose access days. The results for repeated measures factors were considered significant ($p < .05$) only if also significant when using the Greenhouse-Geisser correction for violation of sphericity. Where interactions were significant, between-subject factors were split to explore simple main effects. Supplementary to the main analysis, individual common sucrose days

were analyzed by t-tests (uncorrected for multiple days) and reported under each graph.

Statistics in this chapter compare sucrose intake in grams on common sucrose days. Unlike the previous chapter, here adjustment for body-weight was not necessary because I did not directly compare animals of different ages. As pups get older and gain body-weight, they become able to consume more solution, thus sucrose intake in pups is expected to increase over days (the decreased intake shown in Chapter 2 Figure 2.1 by Pup 4% ED rats from Day 1 to Day 4, and in Figure 2.2 from Day 1 to Day 20, is due to the body-weight adjustment; Appendix A, Figure A1 showed the volume intake).

In all of the following graphs in this dissertation that show sucrose consumption over multiple days, the amount of sucrose solution consumed per day is reported in grams on the y-axis (left, or both left and right).

Phase I. The six common sucrose days were analyzed in 6 Day (Day 1, 4, 7, 10, 13, 16) by 2 Group (ED, E3D) repeated-measures ANOVA.

Phase II. In most of these experiments, Phase II had 10 common sucrose days. I analyzed these days in two 5-day blocks (Block 1: Days 18, 20, 22, 24, and 26; Block 2: Days 28, 30, 32, 34, 36) by 2 separate 5 Day (common sucrose days) by 2 Group (ED, E3D) repeated-measures ANOVAs.

Because measures of statistical significance may not be helpful for understand the practical significance or size of the difference between two groups, effect sizes as measured by partial eta squared (η^2_p) for the between-

group ANOVAs are provided. Effect size measures also allow for comparing the relative difference or impact of treatment across studies that have unequal sample sizes (Keppel, 1991).

Experiment 1: ICP with Adults Given 4% Sucrose

I planned a series of experiments in pups and adults with the ICP using parallel procedures to explore whether pups are relatively invulnerable from developing longer-term changes in sucrose consumption behaviour. Because I was particularly interested in the vulnerability of pups to the ICP, and the pup period is short (~22-39 days of age), the length of Phase I had to be limited in these parallel experiments. As such, in each of these experiments, Phase I consists of five intermittent cycles or 16 days (22-37 days of age in pups). This represents a relatively short Phase I; most experiments with the ICP had 10-15 intermittent cycles in Phase I (Celejewski, 2011; Eikelboom & Hewitt, 2016; Senthinathan, 2012).

This first experiment tests adults with the ICP using 4% sucrose. Work with the ICP has tested adults with 4% and reliably shown a pattern of differentiated sucrose consumption in Phase I that is maintained in a uniform Phase II; however, most studies have used a much longer Phase I. To determine whether a short Phase I can have a long-term influence on sucrose consumption behaviour, I first tested adult rats with the relatively short version of the ICP used throughout this chapter.

Method

Animals. Sixteen adult male Sprague-Dawley rats ~60 days old, weighing about 225g at arrival were used for this experiment.

Procedures. Rats were given 4% sucrose in Phase I. All other procedural details are as described in "General Materials and Methods" section.

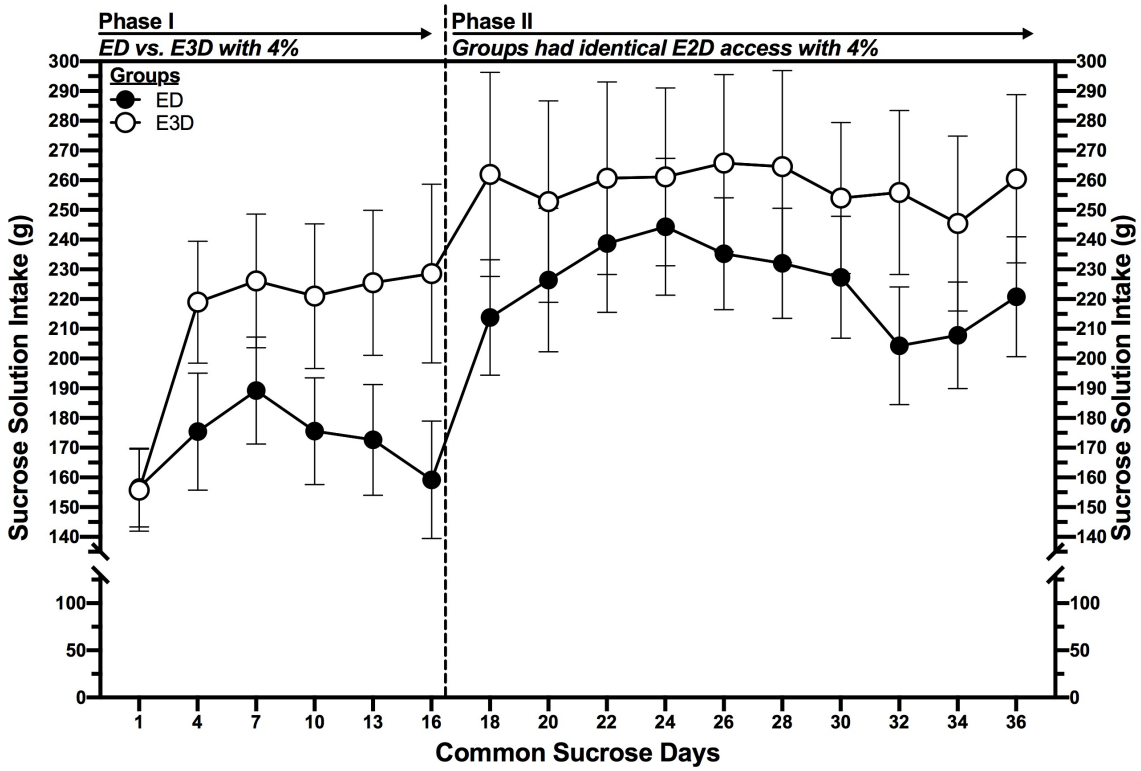
Statistics. As described in "General Materials and Methods" section.

Results and Discussion of Experiment 1

Phase I. On Day 1 the ED group consumed 156.6 ± 13.2 g of sucrose, and the E3D group 155.7 ± 13.8 g (Figure 3.1). Because animals were matched on Day 1 sucrose intake and pseudo-randomly assigned to experimental conditions in order to establish equal groups, no difference in sucrose intake should be present on Day 1. This statement holds true for all subsequent experiments in this dissertation. Analysis of Phase I by a mixed Access by Day (Days 1, 4, 7, 10, 13 and 16) ANOVA revealed an Access by Day interaction ($F(5, 70) = 4.09$, $p = .003$ $\eta_p^2 = .23$), reflective of the developing differences in consumption in the two group shown in Figure 3.1, as well as a Day effect ($F(5, 70) = 9.84$, $p < .001$ $\eta_p^2 = .41$). On the final day of Phase I (Day 16), the E3D rats consumed almost 1.5 times as much sucrose as (~70 g more 4% sucrose than) the ED rats (E3D group: 228.6 ± 30.1 g; ED group: 159.2 ± 19.8 g).

Figure 3.1

Intermittent-Continuous Protocol (ICP) with adult rats given 4% sucrose in Phase I. Mean (± SEM) solution intake (g) for rats receiving solution every third day vs. every day for 16 days in Phase I. Both groups had every second day access to 4% for 20 days in Phase II.



To clarify these results and explore the nature of the interaction in Phase I, I performed two repeated measures ANOVAs for the 6 common sucrose days for each Access group separately. The ED group showed no change across this period, while the intermittent group showed a significant increase in consumption from Day 1 to Day 16 ($F(5,35) = 11.94$, $p < .001$ $\eta_p^2 = .63$). Phase II tested if these

groups would continue to show behavioural differences when shifted to a uniform access schedule.

Phase II. I analyzed Phase II in two separate five-day blocks with mixed 2 Access by 5 Day ANOVAs. Analysis of Block 1 (Days 18, 20, 22, 24, and 26) showed no significant results, suggesting Phase I had no longer-term effect on sucrose intake. A closer look at the pattern of sucrose intake between the groups might suggest otherwise. On the first common sucrose day in Phase II (Day 18), the E3D group consumed about 50 g more sucrose than the ED group, and 8 days later, which was the 5th time that rats were given sucrose in Phase II, the group difference had reduced to about 30 g. To explore this change, I performed two repeated-measures ANOVAs for these 5 days for each group separately. The E3D group showed no change, while the ED group significantly increased their intake on Days 18-26, demonstrated by a significant Day effect ($F(4, 28) = 2.93$, $p = .038$ $\eta_p^2 = .30$). Inspection of Figure 3.1 shows that while the E3D group maintained its elevated consumption, the ED group had a marked increase of sucrose intake (> 50 g) on the first day of Phase II and continued to increase their intake of 4% sucrose across the next 4 common sucrose access days.

Analysis of Block 2 (Days 29, 31, 33, 35, and 37) only revealed a Day effect ($F(4,56) = 3.64$, $p = .011$ $\eta_p^2 = .21$) so intake was not statistically different between the groups. On the final day of Phase II, the E3D group ($M = 260.5 \pm 28.3$ g) consumed ~40 g more 4% sucrose than the ED group ($M = 220.8 \pm 20.1$). As I did for Block 1, I performed two repeated measures ANOVAs for these 5 days for each group separately. Similar to Block 1, sucrose intake by the E3D group

showed no change and the ED group showed a Day effect ($F(4, 28) = 4.40$, $p = .007$ $\eta_p^2 = .39$). Figure 3.1 shows intake by the ED group had a slight dip in Day 32.

The E3D group consumed more sucrose than the ED group throughout Phase I and Phase II. There was an ~70 g sucrose intake difference at the end of Phase I, which immediately reduced to ~50 g on the first day of Phase II, and 18 days later on the final day of the experiment there was still an ~40 g difference. The overall pattern is similar to what we typically observed with adults given 4% sucrose.

Experiment 2: ICP with Pups Given 4% Sucrose

My previous developmental work with the ICP combined the pup and adolescent period so in this experiment the ICP was limited to the pup period and at 39 days all animals were moved to alternate day access. Are pups somehow not influenced by infrequent availability or intermittent access sucrose? Or, are developmental factors such as the size of the small animals and related daily caloric or fluid-volume limits prevent the differentiated consumption difference in these very small animals. This experiment is identical to Experiment 1, but with pups. Here, the 16 days of Phase I span the entire pup period (22-37 days in age). As such, Phase II coincides with the end of the pup period and beginning of adolescence. Given my previous findings with pups, I did not expect any difference in consumption of 4% sucrose in Phase I. Importantly, evidence of any sucrose intake difference in Phase II would suggest that the intermittent access these rats had as pups caused some longer-term change in sucrose consumption behaviour.

Method

Animals. Sixteen male Sprague-Dawley rats aged 21 days at arrival were used for this experiment.

Procedure. Identical to Experiment 1. In this experiment with pups, the common sucrose days in Phase I (Day 1, 4, 7, 10, 13, and 16) spanned the pup period (22-37 days of age). Subsequently, both groups had E2D access in Phase II for 10 common sucrose days.

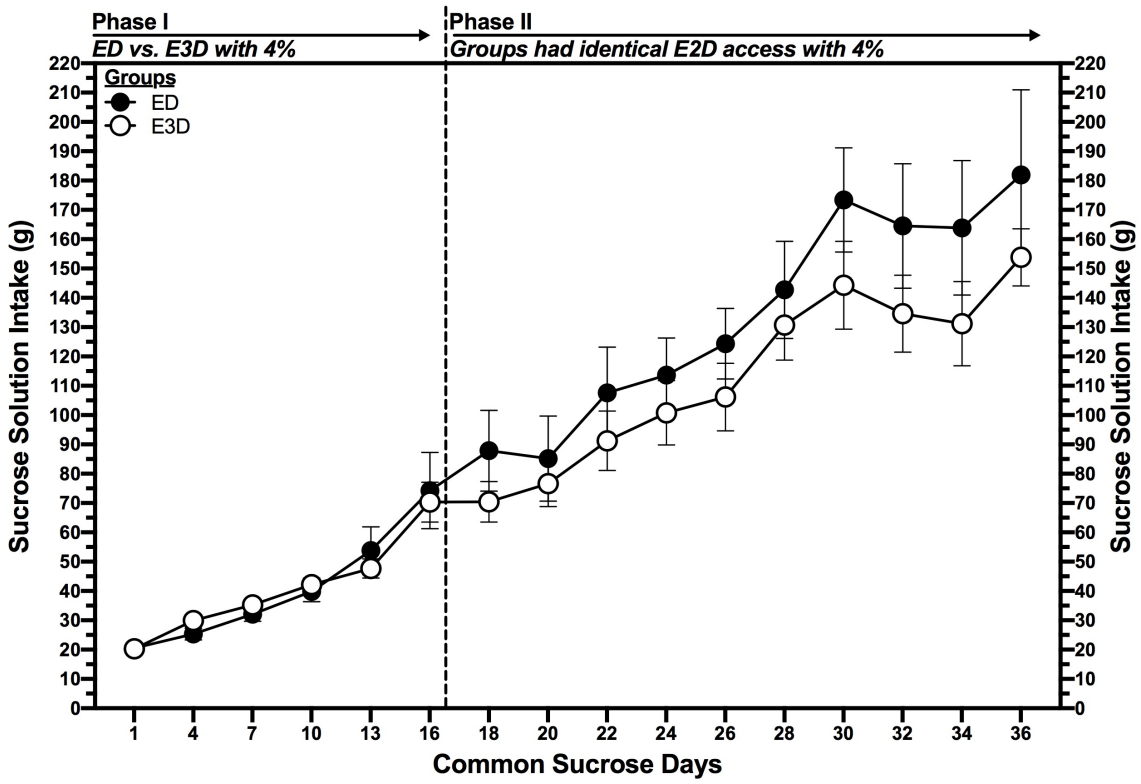
Statistics. As described in “General Materials and Methods” section.

Results and Discussion of Experiment 2

Phase I. On Day 1 the ED group consumed 20.5 ± .9 g of sucrose and the E3D group 20.3 ± .8 g (Figure 3.2). Analysis of Phase I (Days 1, 4, 7, 10, 13 and 16) revealed a Day effect ($F(4,56) = 38.40$, $p < .001$ $\eta_p^2 = .73$), but no interaction or main effect of Access. The groups similarly increased their intake of sucrose across Phase I, and their intake on the final day of Phase I was 74.3 ± 13 g, and 70.3 ± 6.8 g, respectively, for the ED and E3D group. Supporting my previous work with pups (Senthinathan, 2012), no differentiated intake was found in Phase I.

Figure 3.2

Intermittent-Continuous Protocol (ICP) with pup rats given 4% sucrose in Phase I. Mean (± SEM) solution intake (g) for rats receiving solution every third day vs. every day for 16 days in Phase I. Both groups had every second day access to 4% for 20 days in Phase II.



Phase II. Analysis of Block 1 (Days 18, 20, 22, 24, 26) showed only a Day effect ($F(4,56) = 19.20$, $p < .001$ $\eta_p^2 = .58$). Likewise, Block 2 (Day 28, 30, 32, 34, 36) showed only a Day effect ($F(4,56) = 5.19$, $p = .001$ $\eta_p^2 = .27$). These Day effects reflect the increasing sucrose intake by both groups. On the final

experimental day rats were 57 days old, the continuous group consumed 181.9 ± 29.1 g and the intermittent group 153.8 ± 9.8 g of the sucrose solution.

The ICP did not induce demonstrated differences in sucrose intake by pups in Phase I, and analysis of Phase II failed to reveal any underlying differences.

Experiment 3: ICP with Adults Given 16% Sucrose

This experiment tested adult rats with the ICP using 16% sucrose. Previous work with adults given 16% has shown that behavioural difference may not emerge with more concentrated solutions but can be unmasked by giving rats a less concentrated solution in the second phase. To test whether such an effect could be established in the relatively short version of the ICP I had used with pups in Experiment 2, I tested adults with 16% in Phase I, and 4% in Phase II. If this relatively short Phase I with 16% has a longer-term influence on sucrose consumption behaviour, the difference might present in Phase II when rats are given 4%.

Method

Animals. Twenty-eight adult male Sprague-Dawley rats ~60 days old, weighting about 225 g at arrival were used for this experiment.

Procedure. Rats were given 16% sucrose in Phase I and Phase II had only 5 common sucrose days. All other procedural details are as described in *General Materials and Methods section.* Note in all experiments in this chapter, rats were always given 4% as the restricted solution in Phase II.

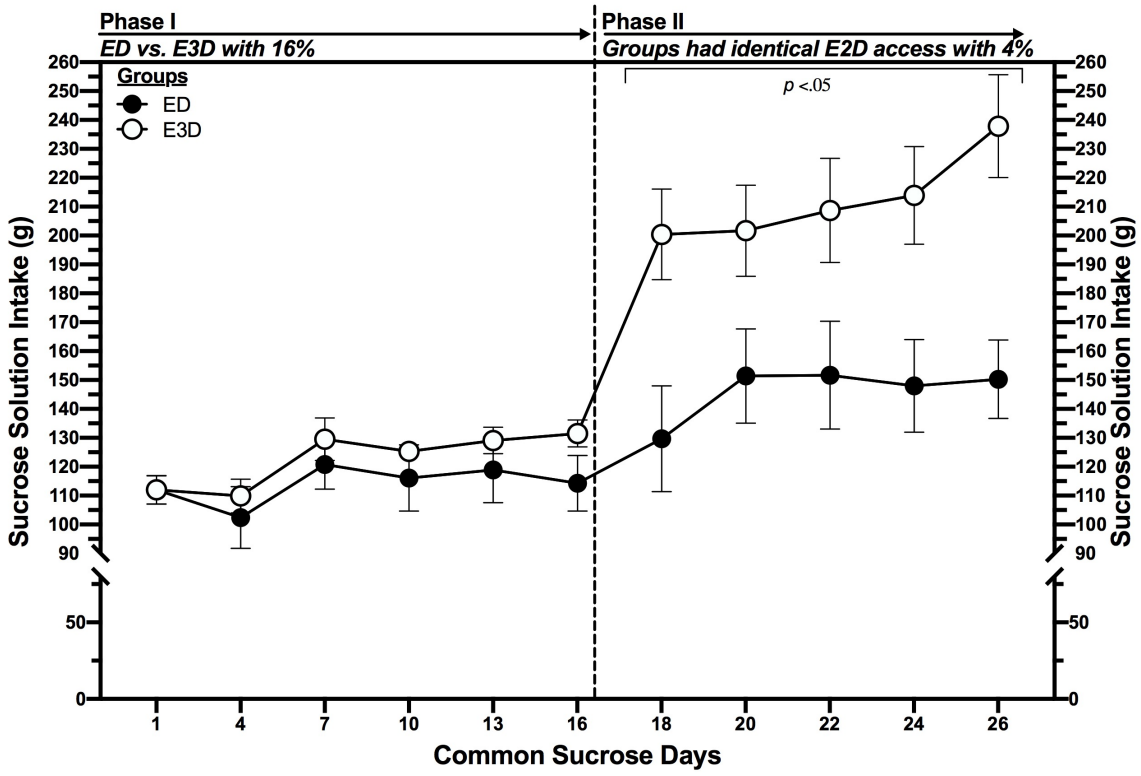
Statistics. As described in “General Materials and Methods” section. Because this experiment had only 5 common sucrose access days in Phase II, analysis of Phase II was done in single 5-day block.

Results and Discussion of Experiment 3

Phase I. On Day 1 the ED group consumed 111.9 ± 4.9 g of sucrose and the E3D group 111.8 ± 4.9 g (Figure 3.3). Analysis of Phase I by a mixed Access by 6 Day (Days, 1, 4, 7, 10, 13, and 16) ANOVA showed a Day effect ($F(5,130) = 5.47$, $p < .001$ $\eta_p^2 = .17$), likely due fluctuating sucrose intake by both groups across Phase I (Figure 3.3). No Access by Day interaction of main effect of access was found, so both groups consumed a similar amount of sucrose across these days. On the final day of Phase I the continuous and intermittent group consumed 114.3 ± 9.6 g, and 131.5 ± 4.7 g of sucrose, respectively.

Figure 3.3

Intermittent-Continuous Protocol (ICP) with adult rats given 16% sucrose in Phase I. Mean (± SEM) solution intake (g) for rats receiving solution every third day vs. every day for 16 days in Phase I. Both groups had every second day access to 4% for 10 days in Phase II.



Note. Supplementary analysis showed the intermittent group consumed more sucrose than the continuous group, on Day 18 $t(26) = -2.93$, $p = .007$, and all subsequent common sucrose days (individual day smallest t-value $t(26) = -2.20$, $p = .037$ on Day 23).

Phase II. Figure 3.3 shows that in Phase II consumption of the 4% solution differed markedly for the two groups with E3D consuming more than the ED group rats. Analysis of Phase II (Day 18, 20, 22, 24, and 26) revealed an

Access by Day interaction ($F(4,104) = 2.97$, $p = .023$ $\eta_p^2 = .10$), an Access effect ($F(1,26) = 8.68$, $p = .007$ $\eta_p^2 = .25$), and a Day effect ($F(4,104) = 6.30$, $p < .001$ $\eta_p^2 = .20$). To explore the nature of the interaction, I performed two repeated measures ANOVAs for the 5 days for each group separately and found a Day effect for the E3D ($F(4,52) = 5.94$, $p = .001$ $\eta_p^2 = .31$) and ED group ($F(4,52) = 2.95$, $p = .028$ $\eta_p^2 = .19$). While both groups increased their intake of sucrose across this period, this increase was relatively greater in the intermittent group (Figure 3.3).

Adult rats given intermittent access to 16% sucrose stably consumed about 120 g of sucrose throughout Phase I, which was about equal to intake by rats with continuous access. There was no apparent difference in sucrose consumption behaviour in Phase I; however, the intermittent exposures to sucrose had an underlying effect that became evident in Phase II. Strikingly, when the groups were switched to Phase II and the rats were given 4% sucrose for the first time (Day 18), a difference seemed to pop out (Figure 3.3). Rats that experienced continuous (ED group) and intermittent (E3D group) access to 16% sucrose in Phase I consumed 150.3 ± 13.6 g and 237.9 ± 17.9 g of 4% sucrose, respectively, on the final day of this experiment (Day 26).

The ICP induced a longer-term difference in these rats. No difference was found in Phase I, but the intermittent vs. continuous access had a lasting influence on sucrose intake. This effect was clearly demonstrated in Phase II when both groups were receiving the same alternate day access to 4% sucrose for 10 days and showed strikingly different sucrose consumption.

Experiment 4: ICP with Pups Given 16% Sucrose

Pups might be invulnerable to the behavioural changes associated with the ICP. Taken together, results from Experiment 2 and 3 support the possibility of some sort of resistance in pups. I continued to explore this by testing pups with 16% sucrose. This experiment is identical to Experiment 3, but with pups and a longer Phase II. To our knowledge this is the first study to test pups with 16% sucrose in the ICP. With this design, will pups present a behavioural difference in Phase II? This would suggest pups are not invulnerable to access-induced behavioural change associated with the ICP. If the ICP does not induce differentiated intake in Phase I with pups given 16%, might it be because of satiety mechanisms or developmental factors that could be unmasked using the 16-4% preparation of the ICP?

Method

Animals. Twenty-four post-weanling male Sprague-Dawley rats aged 21 days at arrival were obtained for this experiment.

Procedure. All procedures were identical to Experiment 1-3 except in this experiment Phase II had ten common sucrose days instead of five.

Statistics. As described in “General Materials and Methods” section.

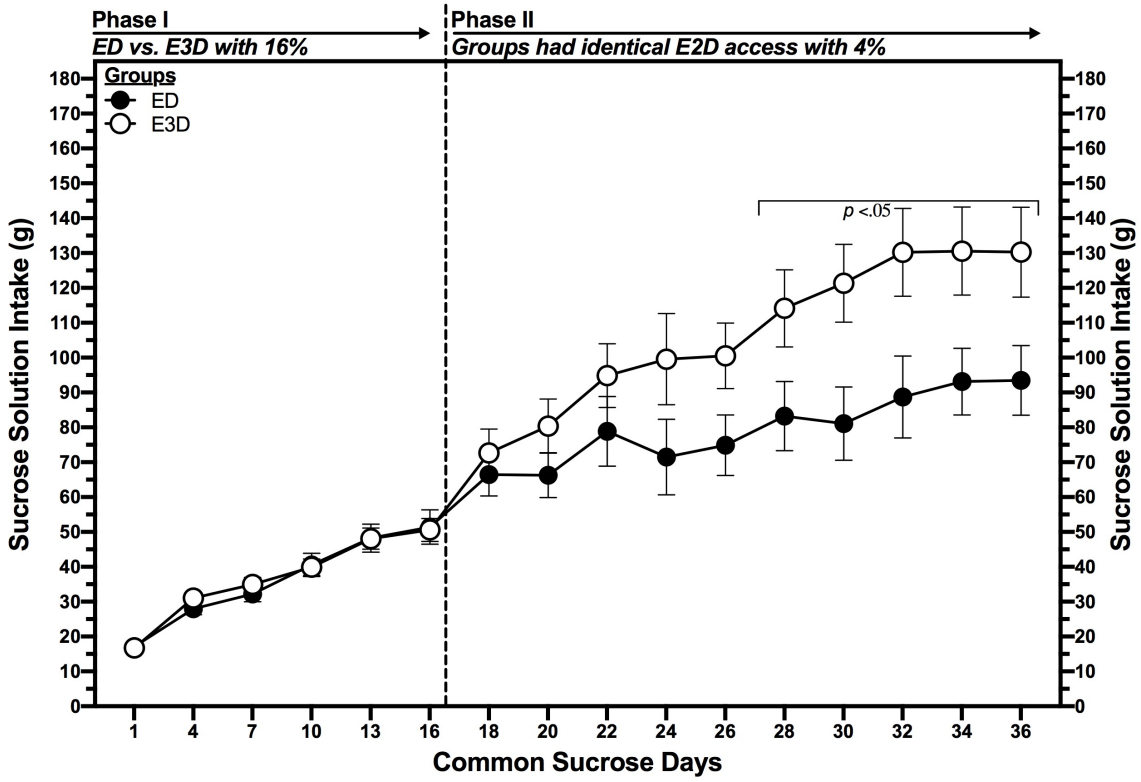
Results and Discussion of Experiment 4

Phase I. On Day 1 the ED group consumed 17.1 ± 1.0 g of sucrose and the E3D group 16.7 ± 1.1 g (Figure 3.4). Analysis of Phase I revealed a Day effect ($F(5, 110) = 89.95$ $p < .001$ $\eta_p^2 = .80$) and no Access by Day interaction or

main effect of Access so both groups similarly increased their intake of 16% sucrose over Phase I. Figure 3.4 shows sucrose intake levels in Phase I were about equal. On the final day of Phase I the continuous and intermittent group consumed 51.4 ± 5.0 g, and 50.6 ± 3.3 g of sucrose, respectively.

Figure 3.4

Intermittent-Continuous Protocol (ICP) with pup rats given 16% sucrose in Phase I. Mean (± SEM) solution intake (g) for rats receiving solution every third day vs. every day for 16 days in Phase I. Both groups had every second day access to 4% for 20 days in Phase II.



Note. Supplementary analysis showed the intermittent group consumed more 4% sucrose than the continuous group on Day 28 (the 6th sucrose day in Phase II), t(14)= -2.08, p = .049 and on all subsequent days.

Phase II. For Phase II, analysis of Block 1 showed a Day effect ($F(4, 88) = 4.30$, $p = .003$ $\eta_p^2 = .16$) but did not reveal an interaction or Access effect suggesting the intermittent exposures to 16% sucrose as pups did not influence sucrose consumption behaviour. Surprisingly, an underlying difference gradually emerged during the second half of Phase II. Analysis of Block 2 showed an Access effect ($F(1, 22) = 5.90$, $p = .023$ $\eta_p^2 = .21$), and Day effect ($F(4, 88) = 6.75$, $p < .001$ $\eta_p^2 = .24$). The Access effect revealed the increased intake in the E3D rats relative to the ED rats while the Day effect in Phase II was reflective of gradually increasing intake across days by both groups (Figure 3.4). On the final day of this experiment, the continuous and intermittent groups consumed 93.5 ± 10 and 130.3 ± 13 g of 4% sucrose, respectively.

The ICP induced a longer-term difference in these pups given 16%. No difference was present in Phase I, but the intermittent vs. continuous access had a lasting influence on sucrose intake. This effect was clearly demonstrated in Phase II when both groups were receiving the same alternate day access to 4% sucrose for 20 days. Surprisingly, the consumption difference emerged in the latter half of Phase II.

Experiment 5: Replication of Experiment 4: ICP with Pups Given 16% Sucrose

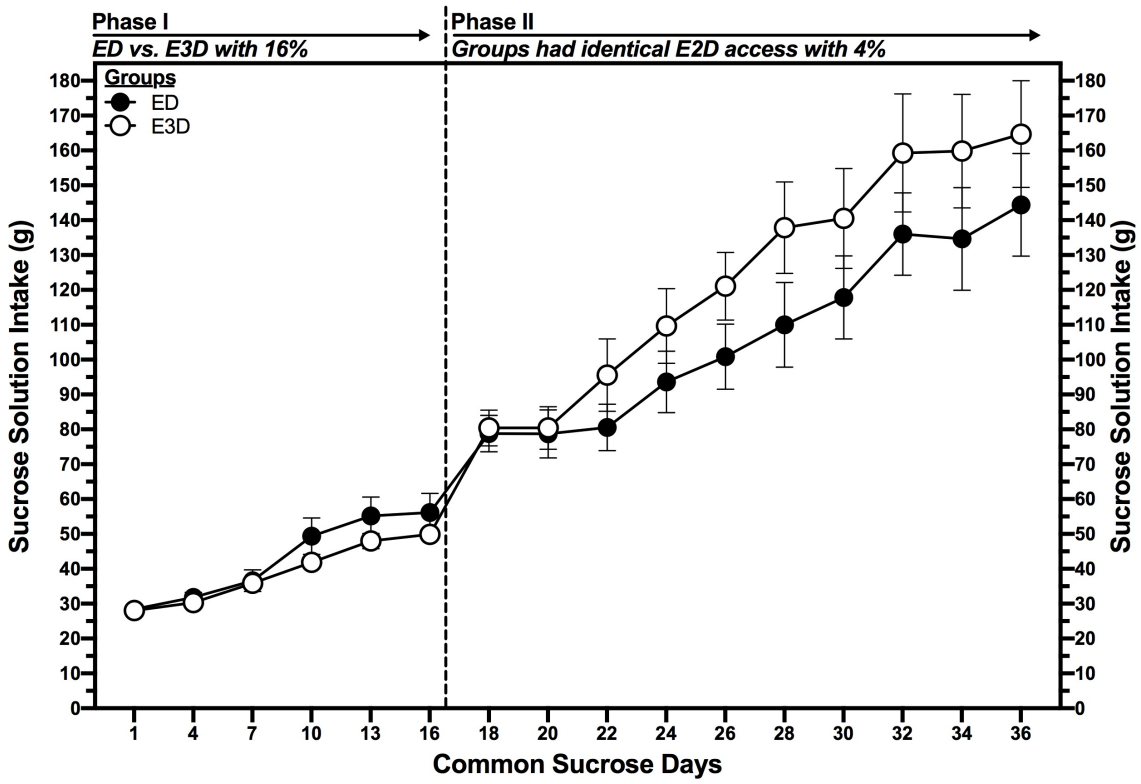
Because Experiment 4 with pups (N = 24, 12 per group) given 16% sucrose in Phase I and 4% in Phase II presented some unexpected results, particularly in Phase II, I chose to replicate the study. All methods and procedures are identical to Experiment 4.

Results and Discussion of Experiment 5

Phase I. On Day 1 the ED group consumed 28.5 ± 1.0 g of sucrose and the E3D group 28.0 ± 1.0 g (Figure 3.5). Analysis of Phase I revealed only a Day effect ($F(5, 110) = 50.55$ $p < .001$ $\eta_p^2 = .70$), suggesting overall, rats increased their intake of 16% sucrose over Phase I. Similarly, Figure 3.5 shows sucrose intake levels in Phase I were about equal. Sucrose intake on the final day of Phase I was 56.1 ± 5.5 g, and 49.9 ± 1.3 g, respectively, for the ED and E3D group.

Figure 3.5

Replication of Experiment 4. Intermittent-Continuous Protocol (ICP) with pup rats given 16% sucrose in Phase I. Mean (± SEM) solution intake (g) for rats receiving solution every third day vs. every day for 16 days in Phase I. Both groups had every second day access to 4% for 20 days in Phase II.



Phase II. Analysis of Block 1 of Phase II revealed an Access by Day interaction ($F(4,88) = 2.96$, $p = .024$ $\eta_p^2 = .12$) and Day effect ($F(4, 88) = 30.57$, $p < .001$ $\eta_p^2 = .58$). To explore the interaction, I performed two repeated measures ANOVAs for the 5 common sucrose days for each group separately. Both groups significantly increased their intake of sucrose across this period (Days effect p

< .001 for each group). The Access by Day interaction is due to greater increasing intake by the E3D group compared to the ED group, and reflective of the developing difference in consumption in the two groups shown in Figure 3.5.

Analysis of Block 2 revealed only a Day effect ($F(4, 88) = 16.38$, $p < .001$ $\eta_p^2 = .43$). A closer inspection of Figure 3.5 shows after the second sucrose day in Phase II intake by E3D rats was shifted upwards from ED rats for the rest of the experiment. This pattern is very similar to what I found in the previous experiment though the effect is much less pronounced in the current experiment.

Experiment 6: ICP with Pups Given 16% Sucrose: The Gap Experiment

Carried out jointly with Kristen Thuringer

This experiment is almost identical to Experiments 4 and 5, but, contrasting these previous experiments, the current experiment has four groups. To explore the gradual emergence of the ICP effect that I found in Phase II of Experiments 4 and 5, and to test the robustness of the effect, a period without sucrose (gap period) was added at the beginning of Phase II. Half of the rats with intermittent, and continuous access in Phase I, respectively, experienced the gap without sucrose, creating 4 groups (E3D; E3D+Gap; ED; ED+Gap). How this gap without sucrose might influence sucrose consumption behaviour compared to groups that would not have the gap was not clear. Work with alcohol (Sinclair & Senter, 1967; 1968) and saccharin solutions (Gandelman & Trowill, 1969; Pinel & Rovner, 1977) has shown rats given access to a solution and then deprived of it for a period increase their daily intake of the solution after the deprivation period, but this deprivation effect (DE) fades with repeated exposures to the solution.

The ICP effect and the DE are similar in that they both demonstrate the increased intake of a given solution following a period of forced abstinence. Evidence from our lab suggests there may be important differences between the DE induced by a single gap and the ICP effect of differentiated sucrose intake induced by repeated intermittent/continuous exposures (Celejewski, 2011).

As with all experiments in this chapter, groups of rats had ED or E3D access to 16% sucrose (Phase I), followed by a period with uniform E2D access with 4% sucrose (Phase II). Differentiated sucrose consumption in Phase II of this procedure is related to access conditions in Phase I, and evidence of an ICP

effect. Additionally, two groups had a gap without sucrose during Phase II. Following this gap, if the +Gap groups consume more sucrose than the respective groups that did not have the gap, it would provide evidence of a DE with sucrose in younger rats. To our knowledge, the DE has not previously been tested in pups.

In Experiments 4 and 5 I found pups given intermittent access to 16% sucrose developed elevated sucrose intake levels in the longer-term. Importantly, this Phase II ICP effect gradually emerged across adolescence, even though both groups had the same access to 4% sucrose throughout adolescence (Phase II). If both groups were restricted from sucrose at the end of Phase I, how would this impact sucrose consumption when sucrose is made available? Would the gap eliminate the access-induced sucrose consumption effect? If the Phase II ICP effect is present after the prolonged gap at the beginning Phase II, it would clearly demonstrate a robust longer-term influence of the ICP in pups.

Method

Animals. Forty-one post-weanling male Sprague-Dawley rats aged 21 days at arrival were obtained for this experiment.

Procedure. Procedures were similar to Experiments 4 and 5, except that this experiment had two independent variables, each with two levels (Access: ED, E3D; Gap: +Gap, no-gap). Additionally, Phase II of this experiment had 15 common sucrose days.

As with the previous experiments, pups had ED or E3D access in Phase I for 16 days with 16% sucrose solution and then sucrose access was shifted to

E2D with 4% (Phase II). The Gap groups (ED+Gap and E3D+Gap) had a 10-day period (or gap) without sucrose, corresponding with the first 5 sucrose days in Phase II (Day 18, 20, 22, 24, and 26) while the no-gap groups (ED and E3D) had E2D access to 4% sucrose. The no-gap groups were identical (replications) to the ED and E3D groups in Experiments 4 and 5. The 12th day of Phase II was the sixth exposure for no-gap groups and marked the day the +Gap groups (ED+gap, E3D+gap) experienced 4% sucrose for the first time (Day 28) after which all groups continued to receive alternate day access for 9 more sucrose days.

Statistics

Phase I. I analyzed Phase I (Day 1, 4, 7, 10, 13, and 16) in a 2 (Access: ED, E3D) by 2 (Gap: +Gap, no-gap) between-subjects factors mixed ANOVA with repeated measures on common sucrose days.

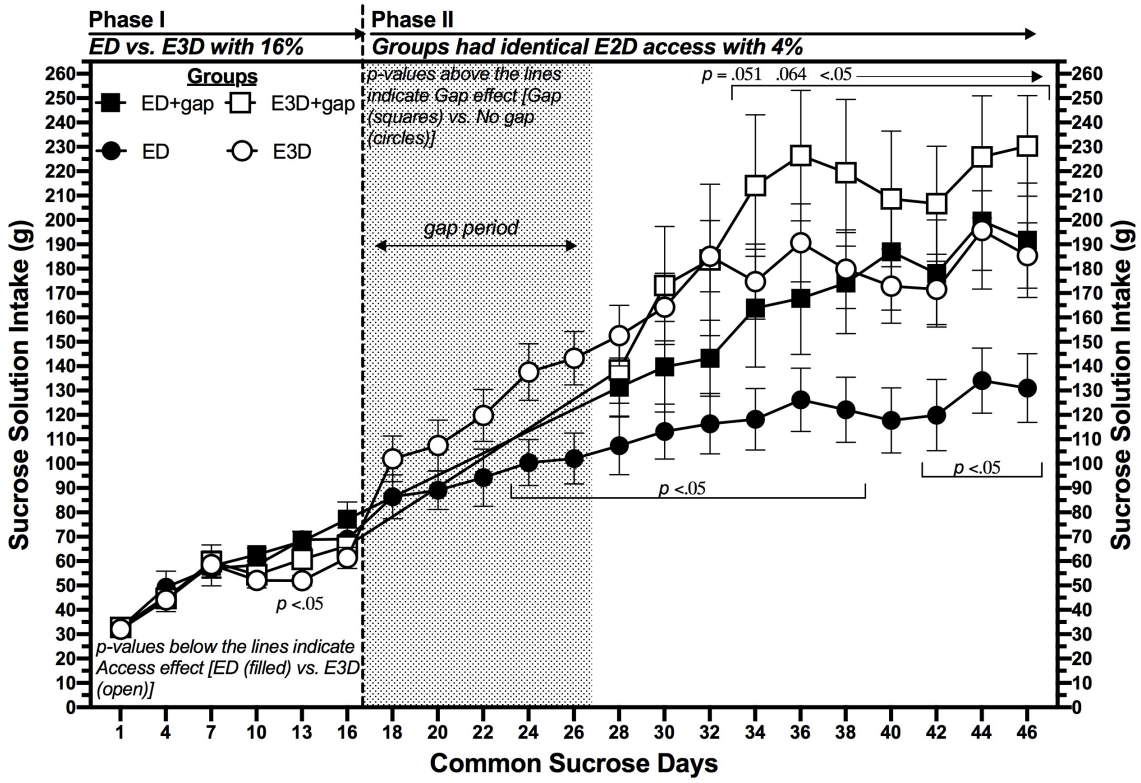
Phase II. Phase II had 15 sucrose days, which I analyzed separately in three 5-day blocks. Block 1 (Day 18, 20, 22, 24, and 26) had only non-gap groups, analyzed in 2 Access by 5 Day repeated measures ANOVA on common sucrose days. Block 2 (Day 28, 30, 32, 34, and 36) and Block 3 (Day 38, 40, 42, 44, and 46) included all experimental groups, analyzed in separate 2 Access by 2 Gap between-subjects factors ANOVAs with repeated measures on common sucrose days.

Results and Discussion of Experiment 6

Phase I. On Day 1 the four groups consumed 32-32.8 g ± 1.5-1.8 g. Figure 3.6 shows all four groups increased their intake of sucrose across Phase I. Analysis of Phase I revealed an Access (ED, E3D) by Day interaction ($F(5, 185) = 2.76$, $p = .02$ $\eta_p^2 = .07$) and Day effect ($F(5, 185) = 63.25$ $p < .001$ $\eta_p^2 = .63$), but no main effect of Access. Unusually, the significant interaction is due to greater intake by ED rats over E3D rats on the last few days of Phase I. Sucrose intake on the final day of Phase I by the four groups was similar, fell in the range between 61-77 g ± 4.3-7 g, and a 2 (Access) by 2 (Gap) ANOVA comparing intake on this day showed no group differences.

Figure 3.6

Intermittent-Continuous Protocol (ICP) with pup rats given 16% sucrose in Phase I. Mean (± SEM) solution intake (in grams, left and right y-axis) for rats receiving solution every third day vs. every day for 16 days in Phase I. Gap groups had a 10-day gap without sucrose following Phase I. All groups had every second day access to 4% in Phase II.



Note. P-values indicate group differences from uncorrected ANOVAs of common sucrose days.

Phase II. Phase II had three 5-day blocks in total (Figure 3.6). The Gap groups (ED+gap and E3D+gap) did not get sucrose for the first 5 sucrose days of Phase II.

Block 1 (Gap period: Results only Relate to No-Gap Groups). Analysis of Block 1 revealed an Access by Day interaction ($F(4,76) = 3.85$, $p = .007$ $\eta_p^2 = .17$) reflective of developing differences, and Day effect ($F(4,76) = 18.84$, $p < .001$ $\eta_p^2 = .50$). The Access effect approached statistical significance ($F(1,19) = 4.10$, $p = .057$, $\eta_p^2 = .178$). To investigate the nature of the interaction I analyzed each Access group separately and only found a significant Day effect in the E3D group ($F(4,40) = 24.61$, $p < .001$ $\eta_p^2 = .71$), demonstrating only this group significantly increased intake of the weaker solution across their first 5 days with 4%. On the last two sucrose days of Block 1 the E3D group consumed significantly more 4% sucrose than the ED group (Day 24: $t(19) = 6.03$, $p = .024$; Day 26: $t(19) = 7.35$, $p = .014$).

Block 2. Analysis of Block 2 revealed an Access by Day interaction ($F(4,148) = 4.13$, $p = .003$ $\eta_p^2 = .10$), Gap by Day interaction ($F(4,148) = 5.85$, $p < .001$ $\eta_p^2 = .14$), main effect of Access ($F(1,37) = 7.44$, $p = .010$, $\eta_p^2 = .167$), and a Day effect ($F(4, 148) = 26.65$, $p < .001$ $\eta_p^2 = .42$).

To explore Gap by Day interaction I split the data by Gap conditions and analyzed the groups without (ED, E3D) and with (ED+Gap, E3D+Gap) the gap separately. The groups without the gap showed an Access effect ($F(1,19) = 9.93$, $p = .005$ $\eta_p^2 = .34$) and the +Gap groups showed an Access by Days interaction ($F(4, 72) = 2.76$, $p < .05$ $\eta_p^2 = .13$) but no main effect of Access. While the non-gap groups were presenting a consistent difference, the effect was emerging in the gap groups. Additionally, both splits showed a Day effect across Block 2 (p-

values < .001) reflecting the general pattern of increased sucrose intake across Block 2 by all the groups (Figure 3.6).

Block 3. Analysis of Block 3 revealed an Access effect (ED, E3D) ($F(1,37) = 5.21$, $p = .028$, $\eta_p^2 = .123$), Gap effect ($F(4,148) = 6.47$, $p < .015$, $\eta_p^2 = .149$), and Day effect ($F(4, 148) = 8.23$, $p < .001$ $\eta_p^2 = .18$), but no significant interactions. This result clearly demonstrates the lasting impact intermittent vs. continuous access to 16% sucrose in Phase I (as pups) had on these rats. As is evident the groups with a gap consumed more than the no- gap groups in this period.

General Discussion

In Experiment 1 adult rats given intermittent access to 4% sucrose increased their intake in Phase I compared to the continuous group and maintained their elevated levels of intake during a Phase II with E2D access. The initial access-induced difference established and evident in the Phase I interaction, as well as the rapid increase in the continuous group following the shift to Phase II is identical to the results reported by Eikelboom and Hewitt (2016), with a similarly short Phase I: Phase II design. Rats with continuous access increase their intake when shifted to alternate-day access; however, the increase from Phase I to Phase II in ED rats seems smaller in experiments with a longer Phase I (Senthinathan, 2012; Eikelboom & Hewitt, 2016). We do not know how long Phase I must be continued before the increase of sucrose intake in the continuous group associated with the shift to alternate-day access is reduced. Experiment 1 showed a relatively short period of intermittent/continuous access to sucrose can result in a longer-term consumption difference.

Experiment 2 tested pups with the 4% sucrose. Phase I spanned the pup period and the groups were shifted to Phase II at the end of the pup period. These pups did not show any differences in Phase I, or as adolescents in Phase II. This result suggested, somehow with weak 4% sucrose solutions pups are resistant to the access-induced changes associated with the ICP. There is a notable difference between this study with pups and the studies with adults given 4% sucrose. Adult E3D groups typically increase their intake across the first few days, while ED groups typically gradually reduce sucrose intake, and this contrasting pattern quickly results in a fairly large sucrose intake difference in Phase I (Eikelboom & Hewitt, 2016; Rehn & Boakes, 2019; & Experiment 1). As such, with adults given 4%, Phase II tests if a pre-established difference will be maintained. In contrast, with pups there is no appreciable sucrose intake difference in Phase I. So, Phase II tested whether a consumption difference would emerge (even though it was not expected or evident in Phase I). To address this inconsistency and test whether just six intermittent/continuous days can have a long-term influence on sucrose consumption behaviour in a design that would mirror the pattern of consumption I found with pups given 4%, in Experiment 3 I tested adults with a more concentrated 16% solution in Phase I and 4% in Phase II, expecting the groups to consume similar amounts in Phase I, but that a difference would emerge in Phase II with a lower solution. If this occurred, it would be important to test pups with the same higher concentration sucrose solution.

Adults given 16% in Experiment 3 consumed similar amounts in Phase I but the ICP effect immediately showed in Phase II with 4%. The group sucrose

intake difference maintained across Phase II showing the effect is robust. Additionally, across Phase II both groups significantly increased their intake of 4% sucrose.

Differences in daily consumption patterns can be difficult to detect with caloric and rewarding solutions. Experiment 3 showed a few intermittent exposures to sucrose could induce a persistent change in sucrose consumption (intake difference in Phase II) even if intake is similar in Phase I. An underlying group difference became evident when all rats were shifted to a common access schedule and the concentration of sucrose was lowered to 4%. Since the sucrose difference emerged in Phase II even though no difference was evident in Phase I, this result from Experiment 3 with adults given 16% adds strength to the findings from Experiment 2 with pups given 4%, which also showed no difference in Phase I. Taken together these experiments suggested pups were resistant to ICP related changes. To our knowledge, no study had investigated the impact of intermittent access to highly concentrated sucrose solution in pups. To this end, and to test whether intermittent access to a more hedonically valuable solution could overcome the suggested resistance in pups, Experiment 4 tested pups with 16%.

Experiment 4 showed pups groups given 16% sucrose consumed similar amounts in Phase I of the ICP; however, the E3D/ED access induced a late emerging behavioural difference. The pattern of change in pups appears to have important differences with results obtained in Experiment 3 with adult rats. With adults given 16% in Phase I and 4% in Phase II, the sucrose intake difference or Access effect was not evident in Phase I, but immediately became evident in

Phase II. In other words, the difference popped out on the first day with 4%. Contrastingly, in Experiment 4 with pups it took some time for the Access effect to emerge in Phase II. The difference gradually emerged even though all rats were maintained on the same access schedule after 37 days of age, and only emerged during mid- to late adolescence. Some developmental mechanisms might have prevented the rats from expressing the behavioural change until later in adolescence. I found these results and the potential explanations surprising. To confirm this finding, I replicated this study in Experiment 5.

Experiment 5 results were consistent with Experiment 4 as the same overall pattern replicated (Figure 3.4 and Figure 3.5). In Phase II of these experiments, from Block 1 to Block 2 the sucrose intake difference became larger, suggesting there was an emerging difference across adolescence. In Experiments 4 and 5, since both groups were maintained on the same access schedule across adolescence, the consistent gradual emergence of a sucrose consumption difference between the two groups is striking. This ICP effect was established in pups, showing pups are not invulnerable to behavioural changes associated with the ICP. Given the ICP effect was not as pronounced in the replication (Experiment 5), for clarity, I opted to replicate the experiment again and extend it to include additional groups to further explore this developmental phenomenon.

In Experiment 6 the intermittent/continuous Access groups were further split to include a Gap condition, creating 4 groups (ED, ED+Gap, E3D, E3D+Gap). All rats were given 16% in Phase I and 4% in Phase II. The +Gap groups were restricted from sucrose for 10 days between Phase I and Phase II.

As with Experiments 4 and 5 the ICP effect gradually emerged in Phase II. The E3D group showed greater increasing intake than the ED group across the first 5 days with 4% sucrose (Phase II Block 1). This interaction is remarkable because it is indicative of lasting change in sucrose consumption behaviour that was established in pups and persisted across adolescence, a pattern also found in Experiments 4 and 5.

Perhaps more remarkable, after the 10-day gap without sucrose the +Gap groups showed the same pattern as the no-gap groups did in Block 1. This comparable pattern following the gap without sucrose clearly demonstrates the ICP influence on pups can be very robust. Gap groups showed a protracted DE, with overall elevated intake by +Gap groups compared to the standard E3D and ED groups. Following the gap, the Gap groups did not show a difference immediately, and the difference gradually emerged over days, suggesting the initial lack of difference in the Gap groups with 4% was due to limits on consumption, and the effect emerged as rats grew larger and were able to consume more 4% solution. Alternatively, the effect might develop in pups but require a few experiences with sucrose following the pup period for the pattern to emerge.

Results from the experiment with pups given 4% suggested there are important differences between pups and adults. The experiments with pups given 16% sucrose might suggest that these differences are related to ontogenetic changes that occur across development. With caution, it seems that the differences I found between pups and adults might be related to a gradual

transition that occurs between weaning and adulthood rather than a developmental switch that suddenly makes adolescents (and older rats) less resistant to the impact of availability on sucrose consumption behaviour. Experiments in this chapter showed similarities between pups and adults, as well as several differences.

Sucrose consumption behaviour in pups and adults is affected by the ICP. In pups, the effect was only observed with 16% sucrose followed by 4% in Phase II (similar to my MSc work, the intake difference emerged and became larger over adolescence). The reason for this concentration, or sweetness dependent effect might involve the difference in reward value of the 4% and 16% solutions. A follow-up study might consider testing pups with the ICP with 16% in Phase I and Phase II.

All the work with 16% sucrose demonstrated with a more concentrated solution developing differences in Phase I may not show as a sucrose intake difference but can be elicited with lower concentration solutions. Continuous/intermittent groups in these experiments consumed similar amounts in Phase I and still developed longer-term behavioural differences and this effect only showed in Phase II with 4%. Presumably, some form of learning underlies the change observed in Phase II, and once learned, this altered profile of sucrose consumption seems resistant to change.

Adult continuous/intermittent groups given 16% sucrose do not typically show differences in Phase I or Phase II. If these adult groups are given 4% in

Phase II, the groups immediately consume different amounts: the difference pops out (Experiment 2). Following the same procedure in pups produces a different result. The sucrose intake difference very gradually emerges. I found this result across experiments 3-6.

In Experiments 3-6 rats experienced a negative shift from sweeter to less sweet solution (16-4% sucrose). Across these experiments, inspection of the Figures (3.3 - 3.6) show volume intake went up following the negative shift to the lower solution. For groups to maintain their intake of sucrose solute (and caloric intake from sucrose) with the 16-4% shift they would need to increase their sucrose volume intake four-fold, which is not likely possible due to kidney capacity. The largest volume increase in these 16-4% experiments was in Experiment 3 with adults, in the Adult E3D group, and less than two-fold. This two-fold increase resulted in an E3D vs. ED group difference immediately following the shift. In the 16-4% experiments with pups, no group differences were found immediately following the shift, and rather, the difference gradually emerged with continued alternate-day access to 4%.

Taken together, the ICP work with adults and pups suggests the reason we do not typically find sucrose volume intake differences with highly concentrated solutions in adults across Phase I is related to calories (Eikelboom et al., unpublished) but the reason we do not see it in pups might be more complicated.

To better understand my results, it might be important to consider some of the earlier work with rats drinking sucrose solutions. Collier and Bolles (1968) showed daily intake of sucrose in adult rats increases as concentration goes up from 1-8% and then decreases with increasing concentration, likely related to satiety effects. Across my experiments it seems volume consumption of 4% and 16% solution was similar in Phase I in pups (Experiments 2, 4-6 but not in adults (Experiments 1, 3). Pups in Experiment 2 given 4% consumed about 20 g on Day 1 and both groups consumed about 70 g on the last day of Phase I. Among the various 16% groups in Experiments 4 to 6 intake on Day 1 ranged from about 17-32 g of the sweet solution and on the last day of Phase I 50-73 g. Adults given 4% consumed about 156 grams of sucrose on Day 1 in Experiment 1 and on the last day of Phase I the ED group consumed about that amount while the E3D group consumed about 230 g. Adults in Experiment 3 given 16% consumed about 110 g of sucrose on Day 1 and on the last day of Phase I the ED group was still about that amount while the E3D group consumed about 130g. The numbers seem to overlap with pups but not in adults. I revisit this in Experiment 1 of Chapter 4.

With caution, the lack of an ICP effect with pups given 4% is not likely due to the taste and related consumption of the 4% solution because volume consumption of 4% and 16% sucrose in Phase I of the experiments with pups was similar. It seems adults consumed more 4% than 16% and pups consumed similar amounts of the two solutions. This might suggest the concentration-consumption curve for sucrose is different in pups. The following chapter has one

large experiment that involves a Phase I with pup and adult groups simultaneously receiving either 4% or 16% sucrose and this age-concentration difference is explored.

In adult rats, sugar solute intake shows a sigmoidal pattern as solute intake increases with concentration to a peak at about 16% sucrose (solute intake effect); thus, I expected rats would consume less sugar solute when shifted from 16-4%. Following a shift from sweeter to lesser sweet solutions, rats show reduced sucrose intake compared to others that do not experience the shift, referred to as the successive negative contrast effect (Flaherty, 1999). In Experiments 3-6, each group experienced the 16-4% negative contrast, and after the shift, each group reduced intake of sucrose solute compared to their previous consumption with 16%, which likely involves limits on kidney capacity. The successive negative contrast effect is a transient learned effect and might have had some influence on sucrose consumption behaviour that gradually faded.

Crespi (1942) showed that rats trained to run a maze for a larger food reward, and then shifted to a smaller food reward condition, ran more slowly following the shift compared to rats that did not have experience with the larger reward. Taken together, the behavioral contrast phenomenon reported with sucrose (Flaherty, 1999) might involve motivational process (e.g. reduced motivation for lowered sucrose concentrations) rather than a simple reaction to a change in the taste of the solution. In my experiments with 16% sucrose in Phase I, on the first day of Phase II rats were shifted from 16-4% sucrose, which might uniquely engage motivation systems compared to studies with 4% in Phase I.

Following this shift from 16% to 4%, adult rats maintained the caloric difference they developed in Phase I (Figure 3.3).

I simultaneously explored the ICP and the DE in the last experiment. The ICP and the DE may both increase consumption of young rats. The processes seem to act independently. With the ICP, intermittent access seems to bump up consumption compared to continuous access (ICP effect). The DE further bumped up sucrose intake in both groups, and the DE effect did not interact with the ICP effect. Similarly, previous work with the ICP in adults showed the ICP effect and the DE act independently with sucrose in adult rats (Celejewski, 2011).

In Experiment 6 the DE became stronger over days in adolescence, which contrasts with previous work that had shown the DE dissipates over days (Gandelman & Trowill, 1969; Pinel & Rovner, 1976; Sinclair & Senter, 1967;1968). It is possible that this difference is due to sucrose access differences (continuous access to solution in older experiments compared to the alternate-day access in Phase II in my work).

I was primarily interested in testing if pups were resistant to developing the Phase II differences we find with adults in the ICP. I tested pups with the ICP to see if pups would develop increased sucrose consumption with intermittent access. On the one hand, I found pups given intermittent access to mildly sweet (4%) sucrose solution did not develop any longer-term change in sucrose consumption behaviour. On the other hand, I found that pups given intermittent access to a sweeter (16%) sucrose solution did develop increased sucrose consumption behaviour. Taken together, it seems pups are less sensitive but not

invulnerable from developing a longer-term pattern of increased sucrose consumption behaviour. These experiments showed several interesting findings and raise questions involving age-related differences with, the ICP effect, the dose consumption curve for sucrose, and the successive negative contrast effect. Future analysis might find some interesting starting points within this data.

The influence of availability on reward consumption is profound; circumstances external to one's control can influence how rewards are processed and ultimately influence behaviour. I found that by increasing the concentration or rewarding value of sucrose, I was able to demonstrate that pups can develop longer-term behavioural changes that may not be expressed until mid- to late-adolescence. This finding has important implications. A simple environmental manipulation can have a profound impact on behaviour that is robust and lasting. Furthermore, absence of behaviour is not evidence of absence; the circumstances that can induce increased motivation for sweets or other rewards may not be overt because the influence of environment on an organism may not manifest immediately.

Once the ICP effect develops in Phase I it seems fairly robust and lasting. Some form of learning must underlie the longer-term change. We had not previously explored how the increased sucrose consumption behaviour caused by intermittent access is supported in the brain. In the following Chapter, I describe experiments in which I used Fos-immunochemistry to explore neural activation associated with sucrose intake; these studies are our labs first attempt

to explore neural activation induced by sucrose. The behavioural portion of the Fos experiment parallels Phase I of experiments 1-4 in this chapter.

Limitations and Future Considerations

I found that intermittent access to 16% sucrose can have a profound impact on pups, and effect was not observed with 4% sucrose. With adults, intermittent access to 4% and 16% sucrose produced a similar effect. With caution, one explanation for the discrepant findings with pups and adults is that for pups, the value or rewarding properties of 16% sucrose is similar to less concentrated solution (e.g. 4%) for adults. To explore this, future experiments can test pups and adults with various concentrations of sucrose, and sucrose + artificial sweetener solutions. For example, if 4% plus some amount of a particular artificial sweetener is equal to 16% sucrose (hedonically, as measured by volitional consumption), this mixture of sucrose plus sweetener could be used to test pups. Procedures such as this could shed light on some of the results I have obtained. Notably, differences in the rewarding and aversive properties of artificial sweeteners between pups and adults might still make interpreting the results challenging.

Sucrose intake satisfies part of the rat's energy and water requirements, and this is associated with proportionately reduced chow and water intake. The changes in sucrose consumption I found in rats with continuous vs. intermittent access to sucrose are likely related to differences in motivation and the rewarding value of sucrose between the groups, but I did not test these possibilities directly.

Chapter 4: ICP in Adults and Pups - Fos-Immunoreactivity and Complex Network Analysis

Work in this chapter parallels and extends the ICP experiments in Chapter 3 with 4% and 16% sucrose. Across several experiments with adults and pups, I found the ICP can induce longer-term sucrose intake differences at both developmental stages. In all experiments (except with pups given 4%), intermittent vs. continuous groups developed longer-term behavioural differences in Phase I that were not always evident in this phase, but Phase II showed there was a difference which was robust and resistant to change. Presumably, some form of learning in Phase I underlies the Phase II difference, and consequentially, neural differences related to sucrose intake between the groups should be expected. Our lab has not previously examined the underlying neural differences associated with the Phase II ICP effect. Work in this chapter is largely exploratory and aims to supplement the findings from previous chapters. I used an immunochemistry technique to uncover activity of an immediate early gene (IEG) in the brain. A brief description of this IEG and its use in research is provided below (a more complete description is found in Chapter 1).

Immediate Early Gene: c-Fos

Genes that are rapidly and transiently activated in response to a wide variety of cellular stimuli are classified as IEGs. Broadly, IEGs contribute to long-term changes in neural plasticity; the nerve cell's ability to show acute or long-lasting phenotypic changes in response to external stimuli or cellular processes

(Herrera & Robertson, 1996). Several IEGs have been identified, with different time courses for expression. The IEG *c-fos* is among the most widely studied and best characterized (Herrera & Robertson, 1996).

The immunoreactivity (IR) of Fos (the protein product of the gene *c-fos*) can be localized to every distinguishable structure within the brain. The expression of Fos is activity-dependent (Sagar et al., 1988). The term “Fos-expression” is used throughout this dissertation to describe localized c-Fos (protein) expression, which reflects nerve-cell depolarization. Fos protein is not usually detectable in most brain areas but is rapidly induced in response to various stimuli (Hughes et al., 1992; McReynolds et al., 2018). Consequently, the quantification of Fos is a powerful tool for exploring neural activity because the protein can be used as biological marker of recent cellular activity.

Experiment 1: Pups and Adults Given ED or E3D Access to 4% or 16% Sucrose for 16 Days: Behavioural Data and Fos Expression

All experiments in Chapter 3 followed the ICP used in Chapter 3 experiments. In Phase I, rats received E3D or ED access to sucrose for 16 days (6 intermittent E3D exposures). Over the six common sucrose days, the adult rats with 4% E3D vs. ED access consumed different levels of solution, with Adult 4% E3D rats consuming much more sucrose than Adult ED rats (Ch. 3 Experiment 1). In contrast, the adult rats with 16% E3D vs. ED consumed similar levels (Ch. 3 Experiment 3). Like adult 16% groups, the pups given E3D vs. ED access to 4% or 16% sucrose, consumed similar amounts with each concentration (Ch. 3 Experiments 2 and 4-6). To explore longer-term behavioural differences that might

have developed in Phase I of these previous experiments, after the six common sucrose days rats were restricted from sucrose for one day, and then given 4% sucrose beginning on Day 18 on a common alternate-day schedule. In Phase II, I found adult E3D rats consumed more than adult ED rats. Likewise, pup E3D rats consumed more than pup ED rats, but for pups the effect was only gradually evident if they received the stronger solution in Phase I.

Because the results from Chapter 3 seemed to show that adults consume more 4% solution than 16%, while pups consume similar amounts of both solutions, I was interested in exploring volume intake of 4% and 16% sucrose by adults and pups. However, the primary purpose of this experiment was to explore how the longer-term differences in pups and adults with the ICP relate to the way sucrose is processed by the brains of these rats. To this end, in the current experiment instead of shifting the groups to a common E2D schedule for Phase II, and coinciding with the beginning of Phase II in the previous experiments (Day 18), rats were given 1 h access to 4% sucrose to induce sucrose intake-related Fos-expression. Differences in Fos-expression could highlight brain areas that are associated with the behavioural differences in sucrose consumption among rats with ED and E3D access.

The 16% groups experienced a sucrose shift from 16% to 4% sucrose. Previous work showed in food deprived rats given 5 min daily access to sucrose (32% or 4%) for 12 days, and on the 13th day given 25 g of sucrose (32% or 4%) solution (32-32, 32-4, 4-4), the downward shift in sucrose concentration (32-4 vs. 4-4) was associated with increased Fos-expression in several brain areas

including the paraventricular thalamus (PVT), paraventricular hypothalamus (PVH), NA_{core}, cingulate, and lateral septum (LS) (Pecoraro & Dallman, 2005), so we might expect more activity in these brain regions in 16% vs. 4% groups, specifically in the ED groups (however, it is important to note the procedures used in this study and ICP work may have important methodological differences).

Methods

Subjects. Sixty-four male Sprague-Dawley rats (32 pups aged 21 days at arrival, and 32 adults ~60 days old) were ordered form Charles River Canada, St. Constant, Quebec in four replications of 16 (8 pups and 8 adults per replication, divided equally into 4 groups per age) and individually housed in conditions as described in previous chapters.

Materials. As described in previous chapters.

Procedures. Daily housekeeping procedures regarding food, water, body-weight measurement, cage changes, etc., were as described in Chapter 3. In each replication, rats were further split to create 8 equal groups (access, age, and sucrose concentration) with a staggered start by one day to facilitate perfusions. Pups and adults were given ED or E3D access to 4% or 16% sucrose for 16 days (6 common sucrose days). Following this phase, on Day 17 rats were deprived of sucrose for 24 h and on the following day (Day 18), given a bottle filled with 60 g of 4% solution for 1 h (about 15 grams of solution will remain in a bottle when the spout has no solution available) to induce sucrose-intake related Fos-expression. Bottles were given to rats one at a time, spaced 30 minutes

apart, in a pseudorandom order across stagger and replications to balance sucrose timing across groups. Fos expression in response to cellular activation is transient, and peaks at 1.5-2 h (Kovacs,1998). To optimize Fos-expression related to the initial taste of sucrose on Day 18, ninety minutes after rats received sucrose (30 minutes after sucrose was removed), rats were anesthetized, perfused transcardially, and tissue was processed for Fos-immunolabelling.

Immunochemistry. Transcardial perfusions were performed with 200ml of 0.1M phosphate–buffered saline (PBS) followed by 200ml of 4% paraformaldehyde in PBS. Following extraction brains were put in 15% sucrose and upon sinking were placed in 30% sucrose phosphate buffer (PB) solution for 72h. Using a cryostat (Leica Microsstemns, Concord, ON) brains were sectioned into 50µm coronal slices and placed in a vial of PB for tissue processing (1) or polyglycerine freezing solution (45% PB, 30% ethylene glycol, 25% glycerol) for storage (4), so that each vial had every fifth tissue section.

Labeling. Tissue from the PB vial was washed in 0.9% hydrogen peroxide for 30 minutes on an orbital stirrer. Subsequently the tissue was given four 15-minute washes in PB, followed by one 30-minute wash in 3% normal goat serum in PB. A 72-hour incubation period followed during which tissue was held at 4 degrees Celsius in a polyclonal c-Fos primary antibody diluted to 1:1000 in phosphate buffered goat serum (0.2%Triton-X 100 in PB, 2% normal goat serum, 0.1% bovine serum albumin). Tissue was then washed for 30 minutes in PB, and subsequently incubated in biotinylated goat anti-rabbit IgG (H+L) antibody (1:5000 in PBGS) for 60 minutes on an orbital stirrer. Tissue was again washed

in PB for 30 minutes and then incubated in ExtraAvidin Peroxidase (1:1000 PBGS) for 60 minutes on an orbital stirrer. Finally, tissue was incubated for 20 minutes in 0.05% 3, 3'-diaminobenzidine tetrahydrocholoride (DAB) with nickel chloride intensification (0.05% DAB, 0.00004% ammonium chloride, .02% L-glucose, 0.02% ammonium nickel sulfate in PB). For visualization, glucose oxidase was added to the DAB solution at 0.5units/ml, with this reaction being stopped after 10 minutes by two washes in PB. All processed tissue was mounted on gelatin-subbed microscope slides, dipped in ethanol to dehydrate the tissue, and cleared using Neoclear solution. The tissue was coverslipped using Permount.

Imaging. Brain tissue was visualized using the Olympus BX43 research light microscope. Brain structures were identified using the rat brain atlas (Paxinos & Watson, 2005). A total of 40 brain areas that could be distinguished from neighbouring structures were included in the analysis. For any given brain area, observations were made at the same coordinates for all brains. Blind to the treatment conditions, an 8 by 6 grid of squares (each square measured 110 μm × 110 μm) was placed over a brain structure and cells positive for Fos-IR were identified and counted. Brain areas were quantified unilaterally, by placing the grid at the center of each brain area, and the data reported represents the total number of Fos positive cells within the grid.

Statistics. Statistical analyses was completed with IBM SPSS version 25.

Behavioural Data. The analysis is very similar to how Phase I of experiments in Chapter 3 was analyzed. The primary analysis was repeated measures factorial ANOVA of the six common sucrose days with three between-subject factors (Age, Access, Sucrose Concentration) and one within-subject factor (Days). The results for repeated measures factors were considered significant ($p < .05$) only if also significant when using the Greenhouse-Geisser correction for violation of sphericity. Unlike experiments in Chapter 3, which had 2 groups balanced by sucrose intake resulting in no initial sucrose intake differences on Day 1, this experiment had multiple groups combined from 4 replications. Also, unlike the previous experiments, which all had a Phase II with common alternate-day access, Phase II in this experiment was a 1 h 4% sucrose test. Day 1 data, and the 1 h sucrose test in Phase II, were both analyzed with separate 2 Access by 2 Age by 2 Sucrose Concentration ANOVAs.

Fos Data. For each of the 40 brain areas explored, Fos expression was analyzed by ANOVA with three independent variables, each with two levels (Age: Pups, Adults; Sucrose Concentration: 4%, 16%; Access condition: ED, E3D).

Results and Discussion: Behavioural Analysis

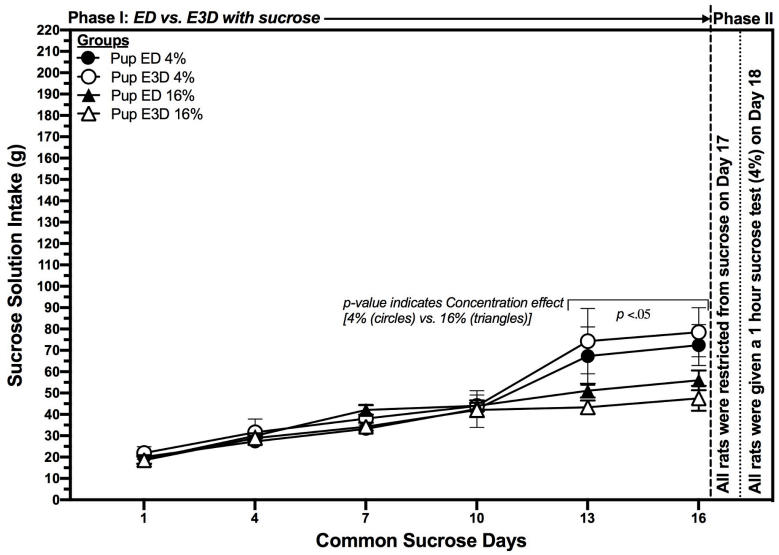
Averaged Day 1 intake by pup 4% groups was 21.0 ± 1.9 g and pup 16% groups 18.7 ± 1.3 g (Figure 4.1). Averaged Day 1 intake by the adult 4% groups was 80.2 ± 12.9 g and adult 16% groups 56.4 ± 1.4 g. A 2 (Access) by 2 (Age) by 2 (Sucrose concentration) ANOVA only demonstrated an Age effect ($F(1,56) = 51.26$, $p < .001$, $\eta_p^2 = .48$) as adults consumed more than pups. Adults are much larger in size than the pups, so it was expected that the adults would consume

more solution. Intake of 4% sucrose was higher compared to 16%, however the difference only approached significance ($F(1,56) = 3.72$, $p < .059$, $\eta_p^2 = .06$).

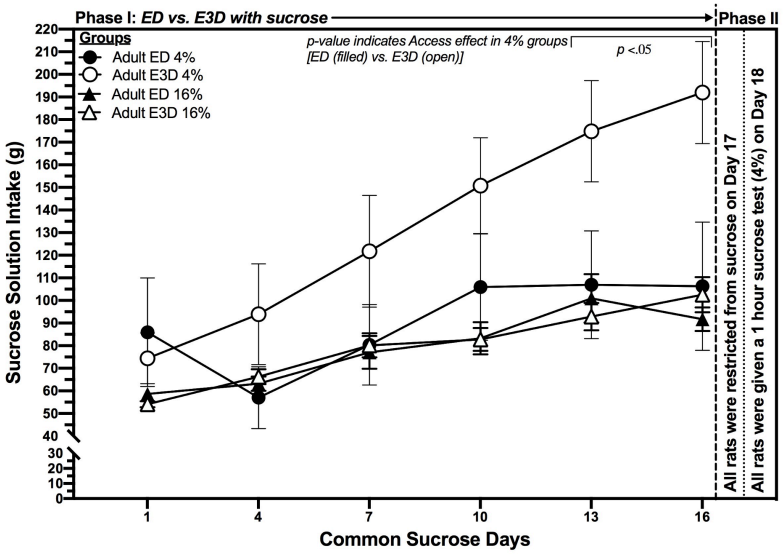
Figure 4.1

Intermittent-Continuous Protocol (ICP) with pup (A) and adult (B) rats given sucrose (4% or 16%) in Phase I. Mean (± SEM) solution intake (g) for rats receiving solution every third day vs. every day for 16 days (Phase I).

A)



B)



I then split the data by Age and separately analyzed the adult (Figure 4.1 A) and pup (Figure 4.1 B) groups across the six common sucrose days.

Adults. Analysis of the adults revealed an Access by Concentration by Day interaction ($F(5, 140) = 2.84$, $p = .018$ $\eta_p^2 = .09$); however, it is not significant when using the Greenhouse Geisser correction ($p = .063$). This analysis also showed an Access by Day interaction ($F(5, 140) = 3.99$, $p = .002$ $\eta_p^2 = .12$), a Concentration by Day interaction ($F(5, 140) = 3.19$, $p = .009$ $\eta_p^2 = .10$), main effect of Concentration ($F(1, 28) = 5.88$, $p = .022$ $\eta_p^2 = .17$), and a Day effect ($F(5, 140) = 27.29$, $p = .001$ $\eta_p^2 = .49$). The results led me to split the adult data by Concentration so the differences could be better understood.

Further splitting the adult data by Concentration (4%, 16%) showed a Day effect for both concentrations ($F(5, 70) = 13.86$, $p < .001$ $\eta_p^2 = .497$; $F(5, 70) = 23.78$, $p < .001$ $\eta_p^2 = .629$ respectively), and an Access by Day interaction only in the adult 4% groups ($F(5, 70) = 3.82$, $p = .004$ $\eta_p^2 = .214$). So as expected, adult rats with intermittent access to 4% sucrose increased solution intake over the days compared to the adults with continuous 4% access, while adults receiving ED and E3D access with 16% consumed similar amounts. Over the days, adult groups with 16% consumed similar levels (they did not show an ICP Phase I effect), while intake by adults with 4% ED was slightly higher than the 16% groups, and the Adult E3D 4% rats consumed much more solution compared to all other adult groups (the 4% groups showed an ICP Phase I effect) (Figure 4.1 A).

Previous work had shown adult rats receiving 4% solution typically consume more solution than adult rats receiving 16% solution (Collier & Bolles, 1968), and over the years in Eikelboom's lab we have found the same pattern. To better understand the data, we looked at intake of 4% vs. 16% sucrose across Day 1 to Day 16 (from my experiments in this dissertation, and data not shown from other previous experiments) and found a reversed pattern in pups and adults. Adults typically showed a difference on Day 1 (ratio 4%/16% = 1.4 to 1.5), which gets smaller (closer to 1) over the days, so the initial difference that had been previously reported (Collier & Bolles 1968) became smaller over days as rats receiving 16% increased intake to match intake by rats receiving 4%.

ICP Phase I Effect. As should be expected given my earlier experiments, adults receiving E3D vs. ED access come to consume different amounts of 4%, but maintain similar amounts of 16%. In other words, adult rats show a Phase I ICP effect with 4%, but not with the more concentrated solution. In our lab, rats given continuous access to 4%, 8%, or 16% solution have all been found to consume about 100 g of solution. In contrast, our ICP work clearly shows rats can consume much more fluid in a day[5]. Over years we have found adult rats receiving 4% sucrose E3D consume between 200-300 g of solution daily (Senthinathan, 2012; Senthinathan & Eikelboom, 2011; 2012; 2013; 2014). The ICP might engage hedonic feeding over drinking.

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[5] Collier and Bolles (1968) noted that even under dire thirst, rats will rarely drink more than 100-110 ml of water daily, which also aligns with what they found with rats receiving 8% sucrose.

Overall, the pattern of intake by the various groups, and the differences between rats that had ED or E3D access (see Figure 4.1) are similar to the results I described across several experiments in Chapter 3. Access did not influence intake of 16% sucrose. Adult rats with E3D access to 4% escalated their intake of sucrose. In contrast, adult rats with ED access to 4% reduced their intake between the first and second common sucrose day, followed by an increase in sucrose intake that seemed to become stable across the fourth to sixth common sucrose day.

Pups. Analysis of the pups revealed only a Concentration by Day interaction ($F(5, 140) = 6.35$, $p < .001$ $\eta_p^2 = .185$) and a Day effect ($F(5, 140) = 48.01$, $p < .001$ $\eta_p^2 = .632$). This interaction in the pups was surprising because across the experiments in Chapter 3, pups seemed to consume similar amounts of the two solutions, thus I only expected a Day effect in the pups. To follow-up, I compared the pup groups on each of the six common sucrose days with 2 Access by 2 Concentration ANOVAs and this analysis revealed a Concentration effect on Day 13 ($F(1, 28) = 5.00$, $p < .05$ $\eta_p^2 = .153$) and Day 16 ($F(1, 28) = 8.00$, $p < .001$ $\eta_p^2 = .224$), demonstrating that the initial intake of 4% and 16% sucrose was similar in pups, and became significantly different by the final two common sucrose days with greater intake of 4% over 16% (Figure 4.1). Thus, the 24 h 16% intake < 4% intake effect gradually emerged in pups over the 16 days. Pups consumed almost identical amounts of 4% and 16% on Day 1 (ratio 4%/16% = 1.1 to 1.2), and the 4%/16% ratio grew larger over days as the pups grew larger

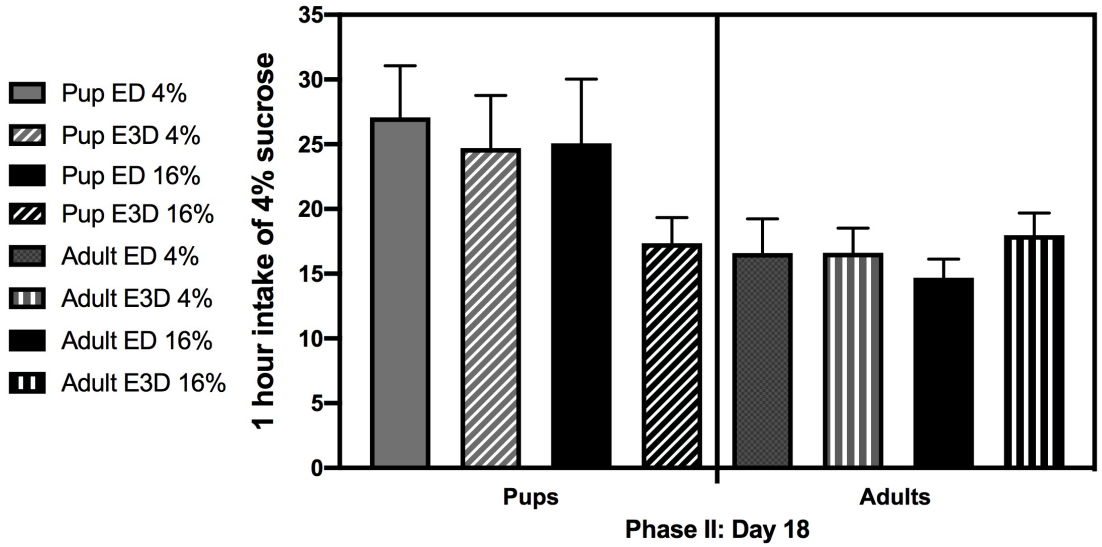
(Day 16, ratio 4%/16% = 1.3 to 1.4). The earlier lack of difference in pups might be related to limits on fluid-volume consumption in pups.

ICP Phase I Effect. As expected, pups given E3D vs. ED access consumed similar amounts (whether 4% sucrose, or 16% sucrose). Overall, with pups, access did not immediately influence intake. Intermittent access vs. continuous access had no influence on intake during the pup period, so pups might be maximizing their intake of each concentration.

Phase II: 1 h with 4% Sucrose. On Day 18, all the rats had 1 h to consume 60 g of 4% solution. Average intake by pups was 23.6 g ± 4.9 g and intake by adults was less, at 16.5 g ± 2.4 g. A 2 Age by 2 Access by 2 Concentration ANOVA revealed only that pups consumed more 4% sucrose in the 1 h sucrose test than adults ($F(1,61) = 10.49$, $p = .002$ $\eta_p^2 = .16$) (Figure 4.2).

Figure 4.2

One hour 4% sucrose intake (g) by all pup (left) and adult (right) groups.



Results and Discussion of Experiment 1: Fos Analysis

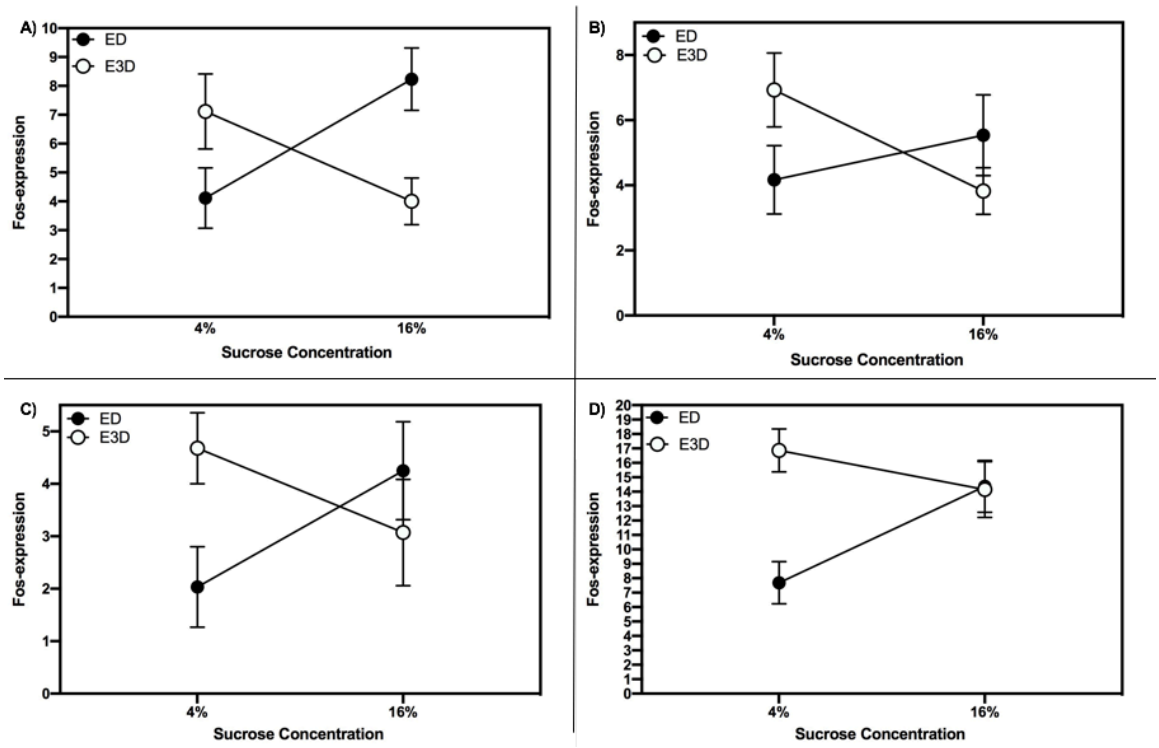
I analyzed Fos-expression in the 40 brain areas with 2 Concentration by 2 Access by 2 Age ANOVAs. Given the large amount of individual comparisons, it is important to note that some differences might be due to chance. Based on previous work (Pecoraro & Dallman, 2005), we might expect a Concentration effect with more Fos-expression in the ED 16% vs. ED 4% groups in the PVT, PVH, NA_{core}, cingulate, and LS. How intermittent access and age of the rats might contribute to differences in sucrose intake related Fos-expression in these brain areas was not clear. The ventral pallidum (VP) is of interest because previous work has shown this structure is particularly involved in palatable food intake (Castro & Berridge, 2014; Covelo et al., 2014; Ho & Berridge, 2013).

ANOVAs comparing FOS expression in the 40 brain areas did not reveal any three-way interactions (full table of raw data in Appendix C).

An Access by Concentration interaction in the ventral part of the LS ($F(1,48) = 11.04$, $p = .002$), the parvocellular part of the PVH ($F(1,49) = 4.05$, $p = .050$), the dorsal cap of the PVH ($F(1,49) = 4.42$, $p = .041$), and the posterior part of the PVT ($F(1,50) = 8.11$, $p = .006$) showed that in rats that had 4% in Phase I, more Fos-expression was found in E3D vs. ED rats, and this pattern is reversed in groups that experienced the shift from 16%-4%, with less Fos-expression in E3D vs. ED groups (Figure 4.3).

Figure 4.3

Fos-expression across rats that received intermittent vs. continuous access to 4% or 16% sucrose in Phase I in the lateral septum (A), the parvicellular part of the paraventricular hypothalamus (PVH) (B), the dorsal cap of the PVH (C), and the posterior part of the paraventricular thalamus (D).



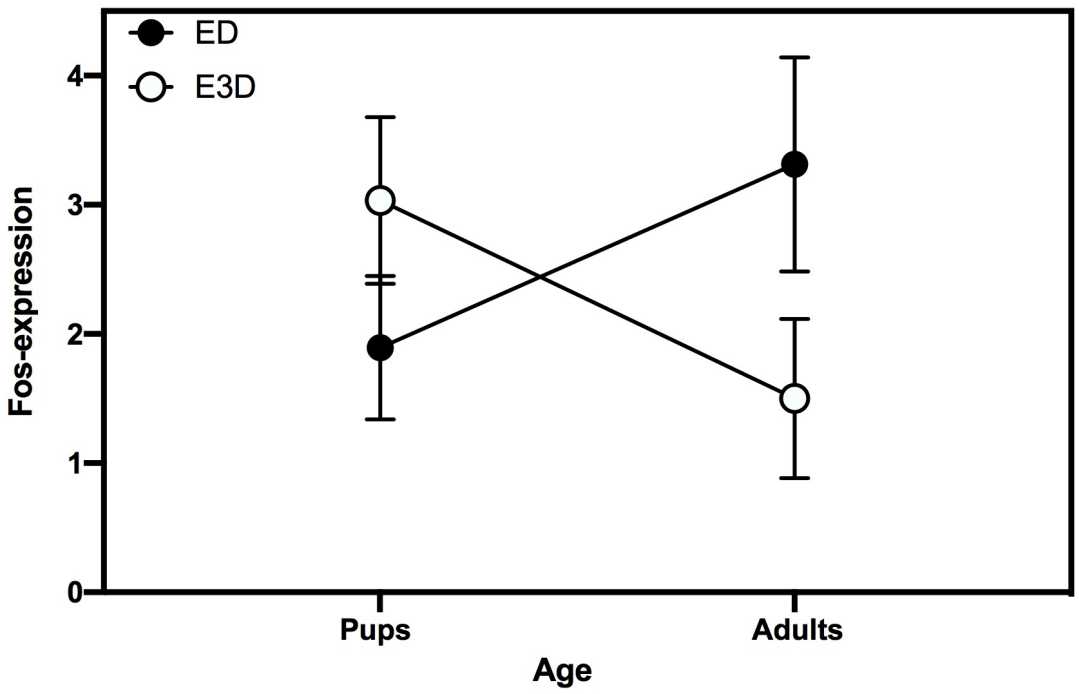
More Fos-expression in the PVT, PVH, and LS was observed in the ED 16% rats compared to ED 4% rats. This is consistent with previous work that showed rats shifted from 32-4% sucrose had more Fos-expression in the PVT, PVH, and LS compared to unshifted rats (4-4%) (Pecoraro & Dallman, 2015). The taste of sucrose along with the negative experience of the shift from sweeter to

less sweet may be engaging these structures more strongly. This effect is reversed in the intermittent group (less Fos-expression in the PVT, PVH, and LS in shifted vs. unshifted groups). Why this effect is reversed in intermittent groups is not clear.

I found an Age by Access interaction ($F(1,52) = 4.12$, $p = .047$) in the VP, reflecting less Fos-expression in the adult E3D groups compared to the adult ED groups, and the reversed pattern in the pups (Figure 4.4). The adult pattern is what was expected based on previous work, and the reason for the reversed pattern in pups is not apparent.

Figure 4.4

Fos-expression across pup and adult rats that received intermittent vs. continuous access to sucrose in Phase I in the ventral pallidum.



In previous experiments (Chapter 3) with the ICP, I found pups that received 4% in Phase I did not develop differences in Phase I or Phase II, and the behavioural difference that developed with pups given 16% in Phase I only showed after many days in Phase II. Therefore, in this experiment, at the age when pups were given the 1 h sucrose test and subsequently sacrificed to explore Fos-expression, I had not previously found any behavioural differences or ICP effects. Given this methodological decision to test pups at the beginning of Phase II, comparing results to the adult groups (who immediately showed a consumption difference in Phase II) is challenging, as it is unclear whether comparable between-group patterns in pup and adult groups that develop the ICP effect should be expected. Giving pups E3D vs. ED access to 16% sucrose in Phase I, and continuing with 4% in Phase II until much later into adolescence, so as to allow the effect to emerge before sacrificing the rats to explore Fos-expression, might provide different results.

It might be important to consider the involvement of the VP and some linked neural circuitry as this structure is known to be involved in consumption of highly palatable foods (Covelo et al., 2014) so it was highlighted as a structure where I expected to find an Access effect. Activation of VP GABAegic receptors influences food intake, with GABA agonists decreasing food intake, and conversely GABA antagonists increasing food intake (Inui et al., 2007; Shimura et al., 2006; Smith & Berridge, 2005). Rats provided a diet containing independent sources of fat, carbohydrate, and protein showed selectively increased fat intake following Intra-VP injection of biculline, a GABA antagonist (Covelo et al., 2014).

In the nucleus accumbens (NAc), the authors report a similar pattern of increased food intake following biculline injection; however, injections to this area did not produce the same selective increase in consumption of the highly palatable fat. Thus, the VP may be uniquely involved in regulating highly palatable foods.

It is unknown what mechanisms led to the differences I found in Fos-expression. In the VP, adults from the E3D group had less Fos expression than adults from the ED group, indicating less cellular activity in the VP. Less FOS-expression (i.e. reduced activity) in the adults that had E3D access (groups that develop a persistent pattern of increased sucrose intake compared to adults with ED) aligns with previous work that suggested a possible difference in the VP between intermittent and continuous groups, because this structure is important for regulating intake of palatable food (Covelo et al., 2014).

The age-related reversed Fos-expression pattern between intermittent and continuous groups in the VP may relate to or play a causal role in the behavioural difference between pups and adults. Given it is well accepted that the relative involvement of a particular brain area to specific behaviours can change developmentally, direct comparisons with pups and adults are not always informative and should be considered with caution. Age-related differences in neural connectivity are likely related to the age related behavioural differences I reported in Chapter 3. Younger rats have fewer projections from the (reward-related) ventral tegmental area (VTA) to the VP compared to adults (Yetnikoff et al., 2014). If the VTA-VP link is important for the ICP difference, and reduced activity in the VP is part of what drives consumption up in intermittent groups, then

a less connected VTA-VP in pups might be related to why pups needed a stronger reward than adults to develop the Phase II ICP effect. This seems to fit with the finding that pups only developed Phase II ICP effect with the stronger 16% solution while adults developed the difference with both a mild 4% solution and 16%.

The VP is intertwined with reward-related neural circuitry (Root et al., 2015) and shares dense connections with parts of the thalamus, including the posterior part of the PVT (pPVT), another area of the brain that shares structural connections with several brain areas involved with feeding, drinking, and other reward related activity. Taste signals from early order taste structures in the hindbrain may reach the cortex via the pPVT (Krout & Loewy, 2000).

The pPVT is particularly involved in reward related feeding behaviour, including situations associated with prediction of food reward (Schiltz et al. 2005; 2007). For example, placing rats in a context previously paired with highly-palatable (highly-rewarding) food resulted in increased Fos-expression in the pPVT, and this effect was not found in rats placed in a context that had been previously paired with (less rewarding) regular rat chow (Schiltz et al. 2005; 2007). In the 4% groups, I found more FOS expression in the pPVT among rats in the E3D groups compared to the ED groups. This aligns with the literature, as we might expect rats on an intermittent E3D schedule to develop food anticipatory behaviour and related neural changes (Mitra et al. 2011). With the 16% groups, Fos-expression in the pPVT was similar between the E3D and ED groups. Since the pPVT is involved in reward anticipation and food reward prediction (Schiltz et

al., 2007), perhaps the taste of 4% (and the related negative shift from 16-4% sucrose) signals that 16% sucrose is not available, and consequently influences activity in the pPVT.

Overall, the Fos data seems to show that more experience with 4% sucrose (continuous access) is associated with less 4% sucrose intake-related Fos-expression compared to intermittent 4% sucrose access. This intermittent vs. continuous Fos-expression pattern is reversed in 16% groups. Continuous access to 4% might devalue this solution compared to intermittent access. Perhaps the experience (Access) by Concentration interaction relates to the anticipation of 16% sucrose by E3D rats, followed by the delivery of 4%, which makes the experience a negative sucrose shift for the 16% groups. If intermittent access vs. continuous access is associated with increased value for the solution, then perhaps the anticipation of 16% sucrose by E3D rats, followed by the delivery of 4% makes the experience more negative for the E3D 16% groups compared to the ED 16% groups.

I identified neural structures that might be involved with differences observed with the ICP. Eating and drinking are maintained by functional neural networks within the brain, so to better understand the neural differences between the groups of rats I tested with sucrose, I explored the functional neural networks associated with sucrose intake and Fos-expression from the current experiment in Experiment 2 by applying the complex network analysis technique to the same Fos-IR data set.

Experiment 2: Complex Network Analysis of Fos-Dataset

Motivated behaviours such as feeding and drinking involve multiple brain regions working in concert. Several techniques can be used to assess large-scale changes in brain activity in humans (e.g. EEG, MEG, fMRI), and functional connectivity among multiple brain regions is typically assessed by covariance of the brain signals. Analogous to this approach in humans, animal studies can employ various techniques to explore neuronal activity and related functional connectivity (Wheeler et al., 2013; Silva et al., 2004). Fos-IR (a measure of neural activity), paired with complex network analysis (a mathematical technique used to explore network parameters), can be used to explore functional neural networks associated with a particular behaviour (Wheeler et al., 2013).

In Experiment 1, I explored differences in Fos-expression related to the consumption of sucrose in pups and adults with varying sucrose experience. Quantification of Fos provided an index of activation for each brain region of interest in each rat. Because sucrose consumption behaviour likely depends on the related activity of individual brain structures (Dela Cruz et al., 2016), I applied the complex network analyses technique to the Fos-IR data-set obtained in Experiment 1 to capture this related activity (functional connectivity) among the brain areas. The discrete Fos analysis in Experiment 1, and the network analysis here, might be exploring very different processes, so we might not expect similar effects across these two types of analysis. While c-Fos expression provides an index of neural activation and has been used in the literature to map out neural activity, c-Fos expression is also involved with long-term changes in synaptic

plasticity and critical for learning and memory (Jaworski et al., 2018). The functional neural networks uncovered by exploring patterns of c-Fos expression among the various groups might reflect differences in learning processes associated with the taste of sucrose.

Application of the complex network analysis technique to c-Fos IR data-sets is a relatively new method for visualizing and conceptualizing neural activation (Wheeler et al., 2013). For an extensive review on complex network analyses see Bullmore and Sporns (2009).

Complex Network Analysis

A neural network is a group of brain structures (nodes) that share connections (edges). These connections can be structural (physical connections, typically through white matter tracts) or functional connections. Functional connectivity relates to the activity levels of two distinct nodes and can be measured through a variety of imaging techniques (Bullmore & Sporns, 2009; Rubinov & Sporns, 2010). Derived from graph theory, a statistical technique known as complex network analysis has been made available and can be used to visualize and explore functional neural networks (Rubinov & Sporns, 2010). Work by the Frankland group demonstrated that Fos activity can be coupled with complex network analysis as a powerful tool for exploring underlying neural network activity (Wheeler, et al., 2013).

In the following experiment, I applied the complex network analysis technique to the Fos-IR dataset from Experiment 1 to visualize and identify

functional neural networks engaged by sucrose intake among the 8 groups of adults and pups with varying sucrose experience. Several properties of functional neural networks were assessed including measures of network integration (connectivity) measured by the number of functional connections (edges) and related network density. Network density is the proportion of possible edges that exist among the nodes of a network. The network matrices were further investigated by comparing “moderate” functional connections with “strong” functional connections. Finally, the modularity of the networks within each of the eight total functional connections matrices, and strong functional connections matrices was explored. Network modularity is a measure of segregation that describes the presence of interconnected groups of brain areas and also determines the exact composition (the networks modular structure) and size of these groups (membership modules). The modularity (community structure) is uncovered by dividing large functional networks into groups of nodes that highlight within-group links. This process typically results in smaller membership modules that have been severed from the larger functional network.

Method

In a 2 Age by 2 Concentration by 2 Access design, pups and adults were given 4- or 16% sucrose ED or E3D (see Experiment 1 for details). For each of these 8 groups (n = 8), Spearman’s rho for the Fos-IR data was calculated for all possible pair-wise combinations of the 40 brain areas. The weight of the correlation coefficient indicates the strength of the functional relationship between a pair of structures. In line with previous work, for all 40 discrete brain

areas assessed in this study, nonparametric spearman rho correlations were used and prior to completion of the complex network analysis all self-connections were removed and any negative correlations were replaced with absolute values (Perit & Mckay, 2012). Network analysis were completed in Matlab using scripts that were adapted from those available online in the Brain Connectivity Toolbox (http://www.brain-connectivity-toolbox.net) (Rubinov & Sporns, 2010). Rubinov and Sporns (2010) described the formulae used to calculate each graph theoretical measure found in this study. Visualization of network structure was completed using Pajek (http://vlado.fmf.uni-lj.si/pub/networks/pajek/) (Batagelj & Mrvar, 2003). Modularity analyses were completed in Pajek using a Kamadi-Kawai separate components algorithm.

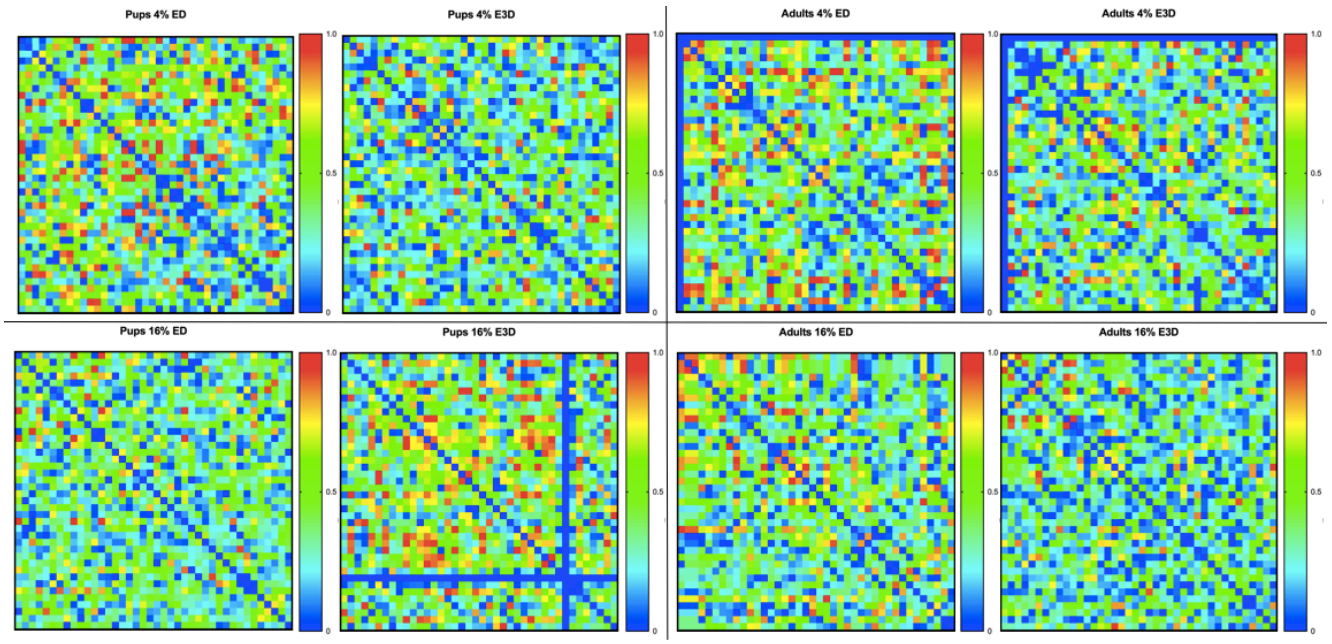
Results and Discussion of Experiment 2

Functional connection matrices were generated for each of the eight groups. Rows and columns in these functional connectivity matrices are composed of 40 nodes arranged rostrocaudally along the y-axis (from top to bottom) and follow the same order on the x-axis (from left to right) (see Appendix C). The order of nodes in these connectivity matrices does not affect computation of network measures. These functional connections matrices are visualized as heat plots. In these heat plots, the individual cells represent the edges within each functional connection matrix, and are shaded such that darker shades are associated with lower rho values and lighter shades are associated with higher rho values. Therefore, the lighter shades reflect stronger functional connections. Below are the 8 heat plots generated for the various groups (Figure 4.5). Visual

inspection of the heat plots reveals a general trend of more “warmer” (lighter shaded) cells among the ED compared to the E3D groups with the exception of Pups given 16% sucrose noticeably showing the opposite pattern. These heat plots represent the network data that is analyzed in subsequent sections.

Figure 4.5

Heat plots for all 8 conditions. Functional connectivity matrices generated from the Spearman rho cross-correlation coefficients for Pups (left) and Adults (right).



Complex network analysis provides objective measures of network parameters; however, interpretation of network data and comparison between groups is not usually approached statistically, and thus is more subjective and open to interpretation. Both age related differences in learning, and how experience shapes the brain at each period, are potential confounds to direct

comparisons between pups and adults. In addition, as noted previously, at the age that pups were sacrificed for brain analysis, no ICP differences had been found. To facilitate interpretation of the network analysis, results from the adults are explored first. Subsequently, data from the pups is described and considered within the context of the results obtained from the adults.

Adults.

Network Density and the Number of Functional Connections. The total number of functional connections and related network density was assessed at several rho threshold values between 0.78 and 0.96 for adults (Figure 4.6). Network density is the ratio of the number of functional connections identified in a network compared to the total number of connections that could exist within the given network. A consistent pattern of reduced network density was found in the adult E3D groups (both 16% and 4%) compared to their respective ED groups while the rats that experienced the negative sucrose shift from 16-4% sucrose (the 16% ED and E3D groups) had reduced network density compared to the unshifted rats.

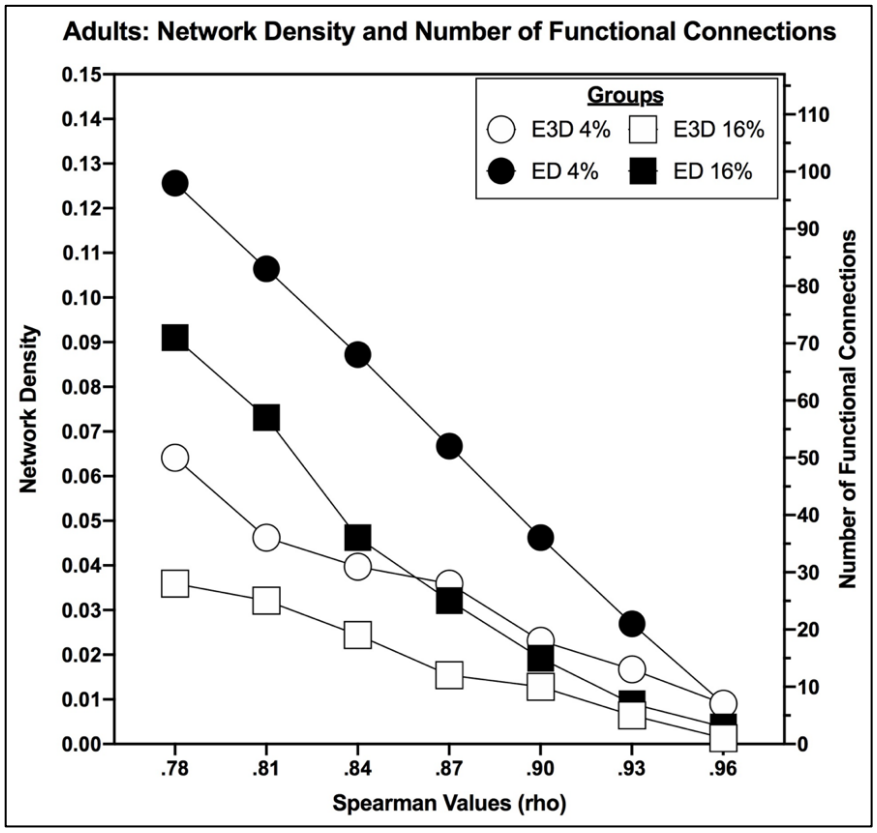
The change in availability for the ED groups from a period of continuous access to a 1-day gap without sucrose might be more salient than the change for E3D groups from intermittent 2-day gaps to a 1-day gap. Continuous groups showed more network density than E3D groups. The switch from 16% to 4% is a noticeable change, while 4% groups did not experience any change in solution. The 16% groups showed less network density compared to 4% groups (Figure 4.6). The relative change, both in sucrose availability, and sucrose concentration

for each group, seems to have oppositely influenced the consistent pattern of reduced network density. Several other possibilities remain, for example, the amount of attention required for regulating consumption may be highest for ED 4% rats because these animals tightly regulated sucrose intake to maintain fairly stable levels whereas the strategy for E3D groups is to maximize intake on sucrose days, and with 16% it is strong enough and rats have already reached maximal intake.

Perhaps the most parsimonious explanation is that the differences in network density are related to the relative value of the 4% sucrose for each group. The network data seems to show that less experience with 4% (E3D 4% group and 16% groups) is associated with less network density following intake of 4%. If ED access to 4% devalues it compared to E3D access, and rats with E3D access come to value sucrose more than rats with continuous access, then reduced network density is associated with increased value for 4%. If what we are seeing with network density is related to the hedonic value of the taste of 4% sucrose, then it seems that greater hedonic value is associated with reduced network density between the brain structures I tested in adult rats, while the negative shift from 16%-4% results in reduced network density, a separate effect.

Figure 4.6

Network density (left y-axis) and total functional connectivity (right y-axis) at increasing thresholds (Spearman's rho values) in adult groups.



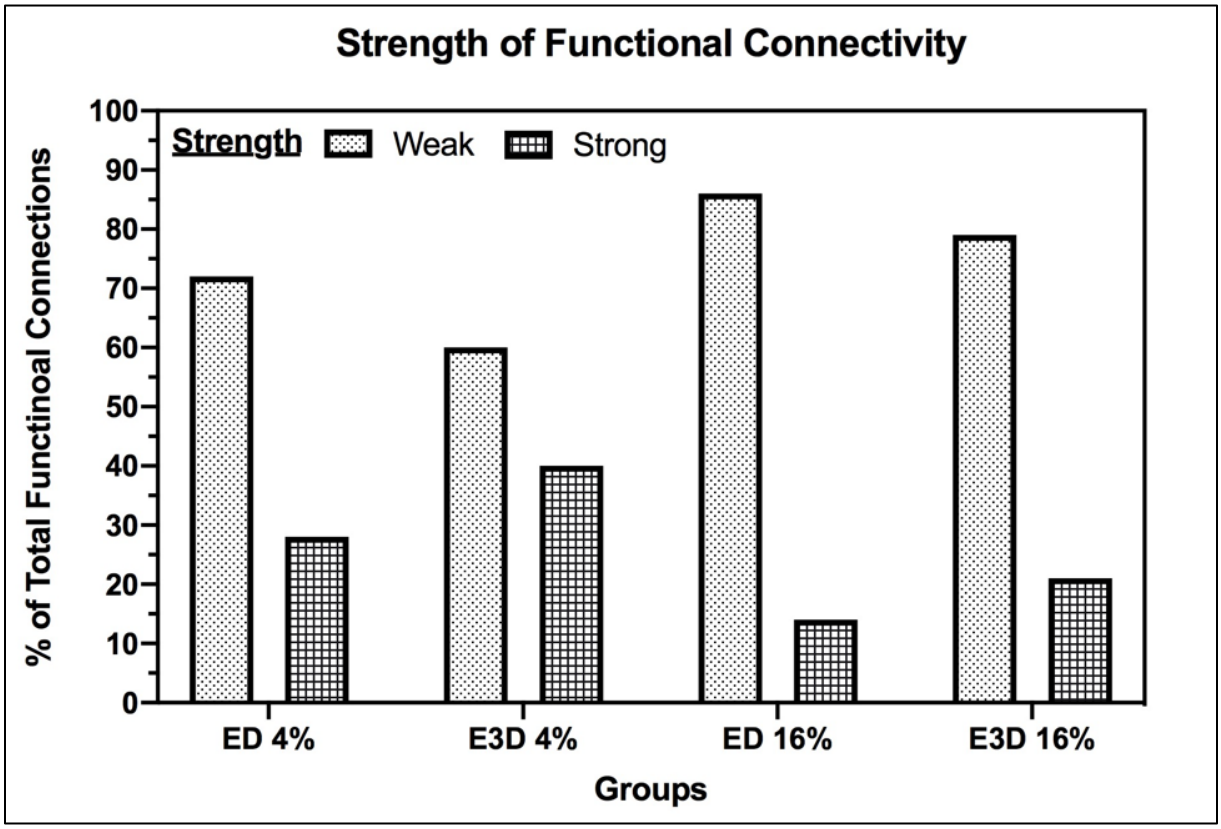
Informed by previous work that investigated functional brain circuits in rats (Perit & McKay, 2012), to further explore functional network connectivity, the following analysis explored moderate, and strong functional connections, defined as connections with rho values ≥ 0.78 ($p < 0.05$) < .93, and rho ≥ 0.93 ($p < 0.01$), respectively. Splitting functional network connectivity into these defined moderate

and strong connections can help identify important changes in functional connectivity that may otherwise not be evident.

Strength of Functional Connections. The strength of functional connectivity was investigated by testing the proportion of moderate to strong functional connections (Figure 4.7). A consistent pattern was found; the proportion of strong to weak functional connections is increased in the adults given intermittent access (both 4% and 16% sucrose), compared to the respective continuous groups. The overall reduced network density in intermittent groups, coupled with the greater proportion of strong to moderate functional connections (i.e. loss of total functional connectivity and network density combined with the strengthening of within network connections) among the intermittent groups compared to continuous groups seems to demonstrate the fine-tuning of a neural network. Overall, the shifted (16-4% sucrose) groups showed a reduced profile of strong to moderate functional connections compared to the unshifted (4-4% sucrose) groups. The overall reduced profile of strong to moderate functional connections in shifted groups seems to be consistent with the network density results, which showed the sucrose shift was associated with less overall network density. One explanation for both the overall reduced network density and reduced proportion of strong to moderate functional connections in shifted groups compared to unshifted groups is that the shift to a weaker solution resulted in weak activation of the system, resulting in less total functional connections overall (Figure 4.6), and fewer strong functional connections to weak connections (Figure 4.7).

Figure 4.7

The relative strength of functional connections in the adult groups.



Results thus far have described the number of functional connections and related network density, as well as the strength of functional connections among 40 discrete brain areas. It is important to consider the organization of these functional connections, which can be approached via analysis of network modularity.

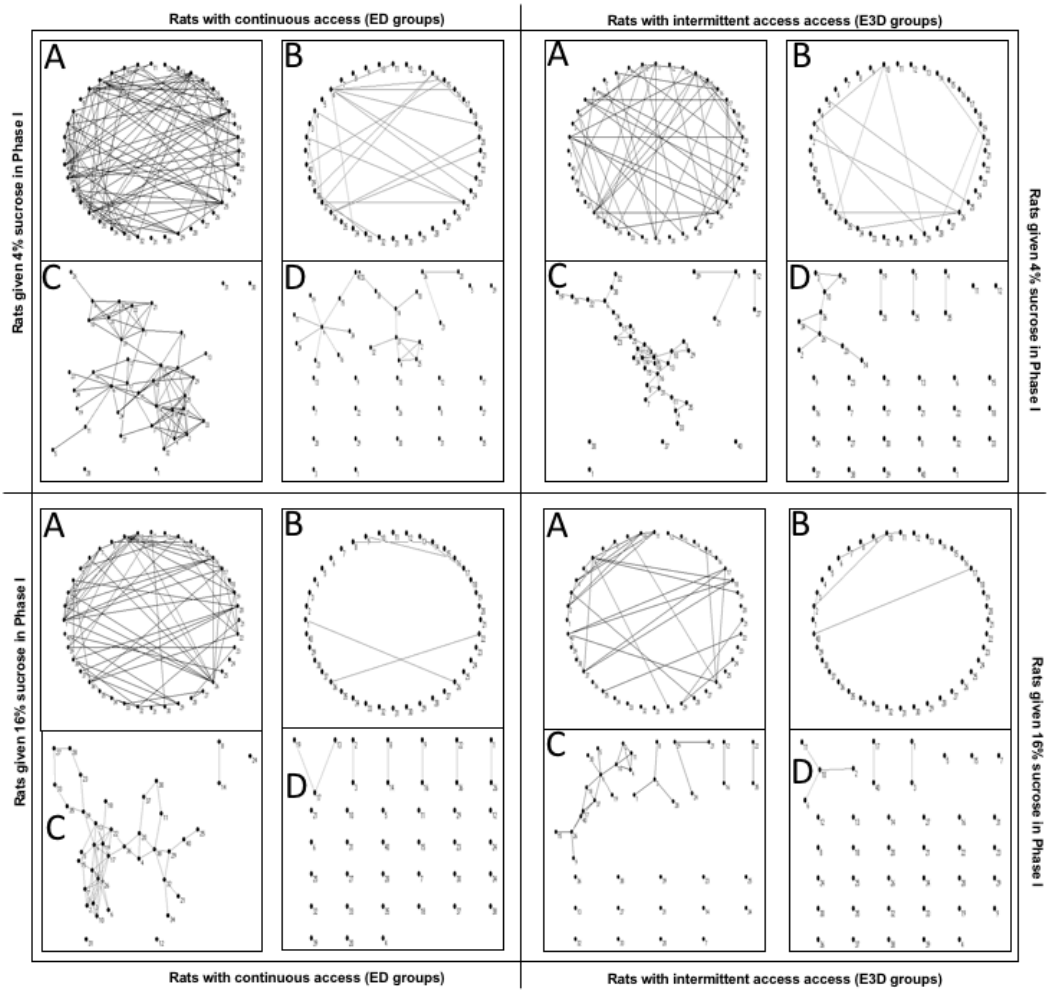
Modularity of the Functional Networks. This final analysis was used to identify and visualize the presence of modules (nodes and their edges) within the

functional connection matrices. For each of the 4 groups of adults, the network modularity was assessed separately based on the total functional connections and strong functional connections (Figure 4.8). I am not aware of a specific way to classify modules by size in a given network. To highlight changes in module structure, I describe small modules as those composed of < 5 nodes (brain structures), medium-sized modules as those with ≥ 5 < 15 nodes, and large modules as those with ≥ 15 nodes.

Analyses of the network modularity based on the total functional connections matrices consistently revealed one large module for each of the groups (and some of these groups showed additional small modules).

Figure 4.8

Modularity analysis. Assessing segregation of functional networks in adults (ED groups on the left, E3D groups on the right, 4% groups on the top and 16% groups at the bottom). The circle-shaped plots show the full networks for the total functional connections (A), and strong functional connections (B). The web-like plots "spider plots" below each circle-shaped plot, shows the respective modular structure of each network (C shows modular structure of A and D shows modular structure of B).



Note. The 40 dots in each panel represent the 40 brain areas.

Network modularity based on the strong functional connections (Figure 4.8 B panels) did not show any large modules in the adult groups, thus, by using this criteria, the larger modules found with the previous analysis were segregated (split and reduced) into smaller modules, highlighting strongly connected networks (only the strong connections will survive while weaker connections are trimmed). This level of analysis can be useful for identifying particularly influential brain structures within a functional network. Investigating the “importance” of a node in a network can be determined by measuring the influence of a given node for the functional performance of a network. The most central or influential brain structures or nodes in a functional network, are known as “hubs” (Sporns, 2013). There is no defined way of identifying these network hubs. To identify hubs within my network data I looked for any nodes that maintained a hub-like structure with at least 5 individual connections that were also not connected to each other with the more stringent modularity criteria (akin to strong connections plots). In the Adult ED 4% group, this analysis revealed a medium sized hub-like module with the dorsal tenia tecta (DTT) at the center (the snowflake-like shape in the strong connections spider-plot, Figure 4.8 D). Compared to other structures in this module, the DTT has a high network degree (number of edges or connections). In subsequent reference to this module in this dissertation, I call it the “DTT-hub”.

In the DTT-hub, the DTT is at the center, with individual connections to the dorsal part of the lateral septum, the ventral part of the PVH, the cingulate gyrus, the lateral part of the substantia nigra, the ventral part of the bed nucleus of the stria terminalis (BNST), the supraoptic nucleus, the piriform area, and the locus

coeruleus. None of the other nodes in this module share any connections with each other, so removal of the DTT from the DTT-hub would result in complete loss of this membership module (interconnected groups of brain structures) while removal of any other node in this module has little impact on the overall architecture. Investigation of network modularity based on the strong functional connections in adult groups did not reveal any other particularly influential hubs.

The DTT-hub may be particularly important for regulating sucrose consumption behaviour in adult rats when it is regularly (continuously) available. The DTT-hub (DTT at the center with individual connections to the brain areas noted above) was not found in the Adult 16% ED group. The lack of DTT-hub engagement in the adult 16% group might be related to the shift from 16-4% sucrose. Future work might explore neural patterns in rats with the ICP using 16% in Phase I and Phase II. Following the same procedure as was done for the adults, the next section considers the results from the pups.

Pups.

Network Density and the Number of Functional Connections. For methodological reasons noted above, interpreting the pup data and/or comparing it to the adults has some challenges. As with the analysis for adults, the total number of functional connections and related network density was assessed at rho threshold values between 0.78 and 0.96 (Figure 4.9). This analysis revealed reduced network density in the pups given intermittent access to 4% compared to the pups given continuous access to 4%. This pattern is consistent with what I found in adults (for both sucrose concentrations). With pups, this analysis

revealed the reverse pattern in 16% groups. In other words, increased network density was found in the pups given E3D access to 16% compared to the pups given ED access to 16%.

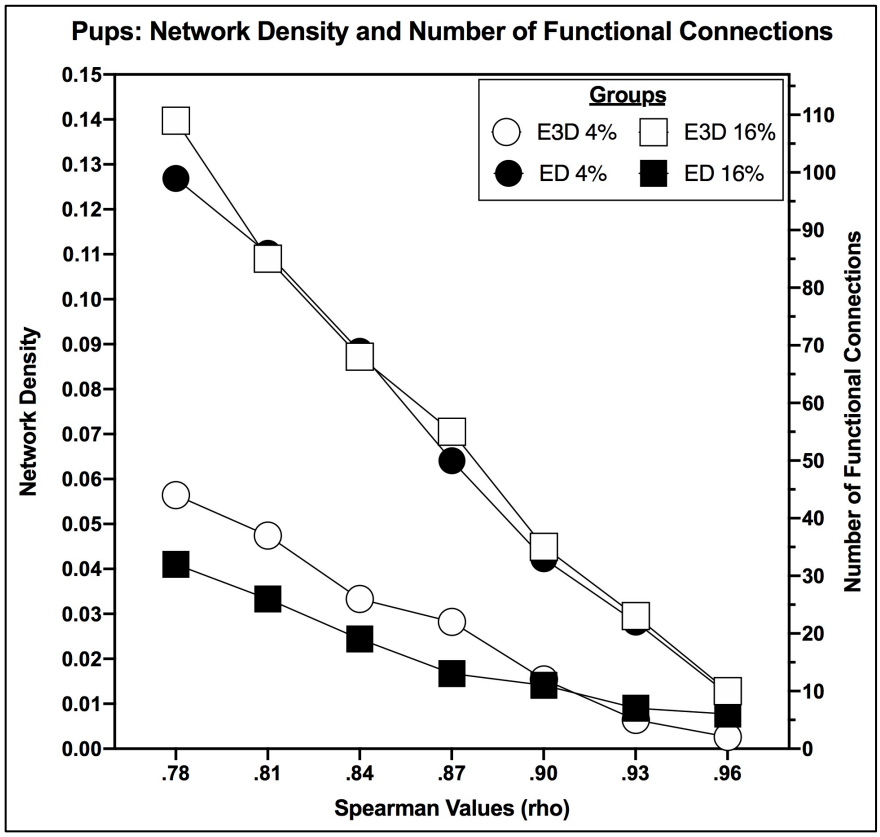
If the ICP behavioural differences mapped on to the network density differences, and we expected some consistency in the network data between the pups and adults, then we might have expected the pup 16% groups to show the adult pattern, and we would not expect the pup 4% groups to show the adult pattern. If the reduced network density is associated with experience with 4%, then we would expect the pattern I found with the pup 4% groups, so perhaps this is the simplest explanation.

With the adults, the sucrose shift for both 16-4% groups seemed to have a consistent effect on network density, with reduced network density compared to unshifted groups. For pups, the sucrose shift effect is not the same consistent downward shift for both 16% groups. The pup 16% ED group showed the downward shift in network density, but the pup 16% E3D group showed the most network density of all the groups. The shift from 16-4% sucrose is likely not the same type of negative experience for pups and adults. It could be argued that the shift from 16-4% sucrose is more negative for pups because pups naturally tend to prefer sweeter solutions. For 16% intermittent pup rats it is particularly negative because they have come to value their solution more than continuous rats, making the shift to a different and lower solution even more negative.

A closer inspection of the data reveals the number of functional connections in adult 4% and pup 4% groups (both ED and E3D) is similar. With the pup 16% groups, the number of functional connections is at the extremes (highest and lowest levels) of the pup network density data. The most functional connections were found in the pups given E3D access to 16%, and the least number of functional connections was found in the pups given ED access to 16%. These patterns are clearest at the lower rho values. The particularly extreme negative shift for the E3D pup 16% group might underlie the network density results. Perhaps in this group the pronounced negative experience engages alternate mechanisms which in turn engage this system, resulting in more total functional connections and greater network density compared to the other groups.

Figure 4.9

Network density (left y-axis) and total functional connectivity (right y-axis) at increasing thresholds (Spearman's rho values) in pup groups.



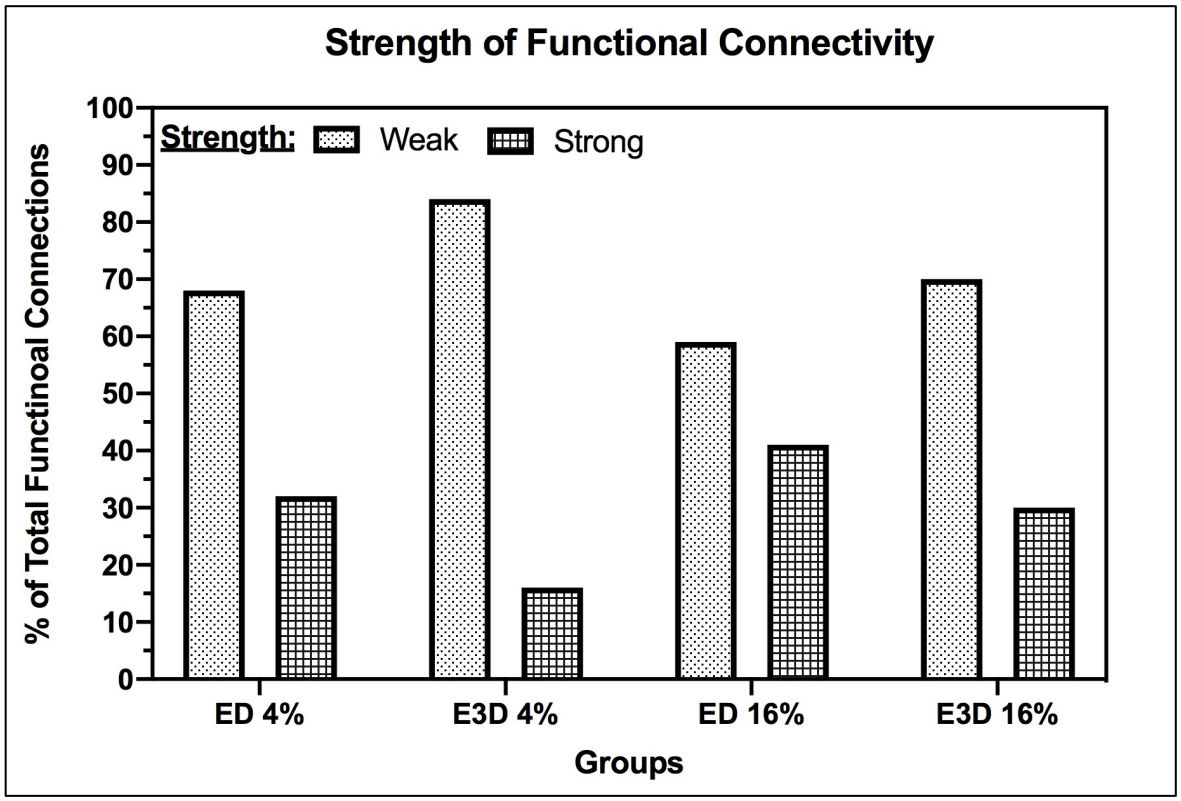
Strength of Functional Connections. Compared to the respective ED groups, pups given E3D access to 4% and 16% sucrose showed a decreased proportion of strong to moderate functional connections as did the 4% compared to 16% (Figure 4.10). This contrasts with the results I obtained with adults (with adults, E3D access was associated with an increased proportion of strong to moderate functional connections). In fact, in the pups the pattern was a complete

reversal of what was found in adults. This highlight that patterns in network topology may only be uncovered when comparing functional connectivity at higher thresholds (rho values).

With adults I suggested that the overall reduced network density in intermittent groups, coupled with the greater proportion of strong to moderate functional connections (i.e. loss of total functional connectivity and network density combined with the strengthening of within network connections) in adult E3D groups compared to adult ED groups seemed to demonstrate the fine-tuning of a neural network. I found the opposite pattern in pups. The reason for this is not clear but might relate to, or play a causal role in why we don't see the ICP effect with pups given 16% sucrose in Phase I until much later in adolescence. Age-related differences in experience-dependent neural plasticity are likely involved.

Figure 4.10

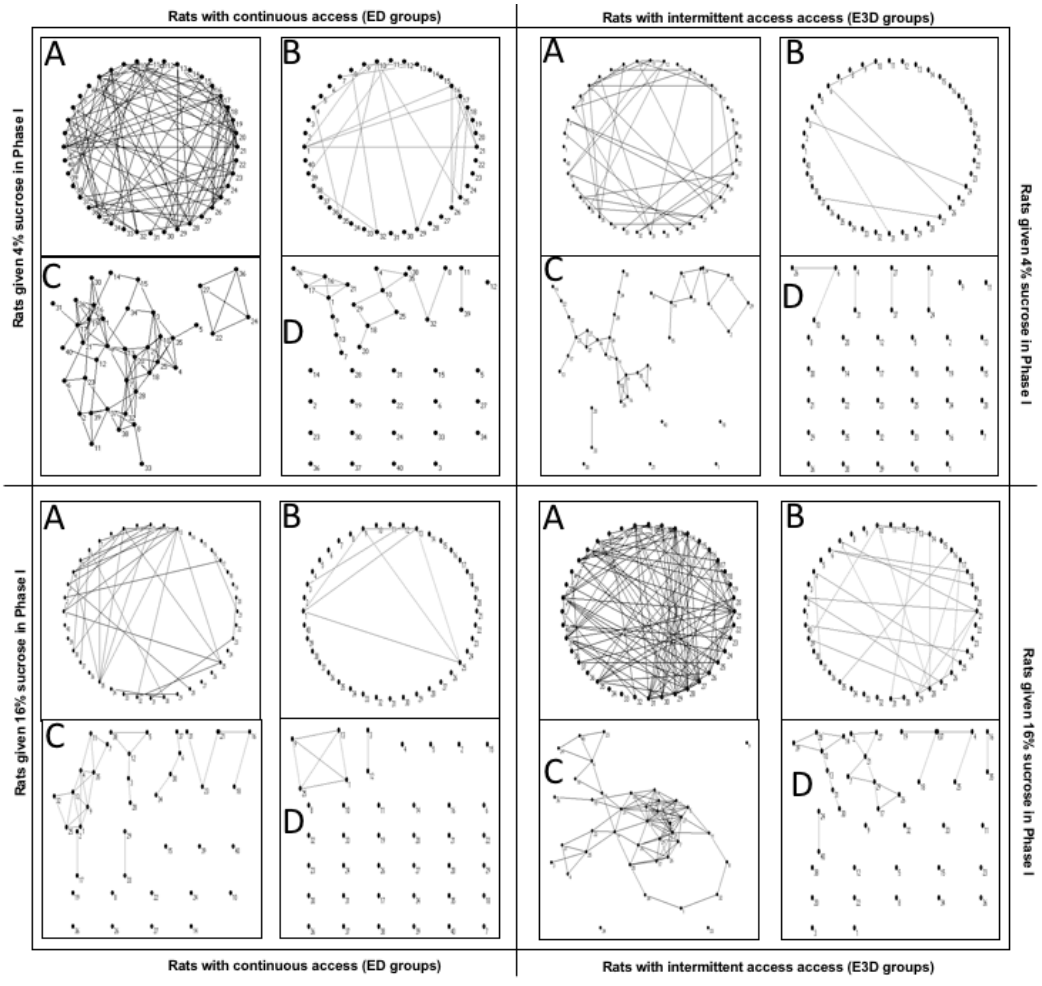
The relative strength of functional connections in the pup groups.



Modularity of the Functional Networks. As with the adults, for each of the 4 groups of pups, network modularity was assessed separately based on the total functional connections, and strong functional connections (Figure 4.11). Analyses based on the total functional connections matrices consistently revealed one large module for each of the groups (and some of these groups showed additional small modules). This pattern is consistent with results from the adults. However, for the pups given ED access to 16%, no large module was present. Only two medium sized modules and four smaller modules were found.

Figure 4.11

Modularity analysis. Assessing segregation of functional networks in pups (ED groups on the left, E3D groups on the right, 4% groups on the top and 16% groups at the bottom). The circle-shaped plots show the full networks for the total functional connections (A), and strong functional connections (B). The web-like plots "spider plots" below each circle-shaped plot, shows the respective modular structure of each network (C shows modular structure of A and D shows modular structure of B).



Note. The 40 dots in each panel represent the 40 brain areas.

Network modularity based on the strong functional connections did not show any large modules in the pup groups, thus, as with the adults, by using the more stringent criteria (higher Rho threshold) to determine functional connectivity, the larger modules found with the previous analysis were further segregated. Investigation of network modularity based on the strong functional connections did not reveal any particularly influential hubs in the pup groups.

I explored neural activation in response to intake of 4% sucrose, which might be considered a novel solution for rats in the 16% groups, and a negative shift from sweeter to less sweet. The DTT-hub might be important for regulating sucrose intake in adults (and not pups). This might explain why the DTT-hub was strongly activated in the Adult 4% ED rats, and not found in the pup ED 4% groups. Testing these rats with 16% in Phase II, instead of 4%, could help to further understand these findings (see limitations).

General Discussion

Work in this chapter is our lab's first attempt to explore neural activity associated with the sucrose consumption differences induced by the ICP. This chapter represents a starting point from which future experiments can continue to expand (see "limitations and future considerations"). Fos-IR (Experiment 1) and complex network analysis (Experiment 2) was used to explore neural activity associated with the intake of sucrose among pups and adults with varying sucrose experience. Each analysis provided some insight into the neural processes that underlie the behavioural differences we typically observe with the ICP.

Because the work in this chapter extends from the experiments in Chapter 3, neural differences should be considered within the context of the previous behavioural data. In Chapter 3 I found adults given Phase I E3D/ED access to 4% or 16% sucrose, showed longer-term sucrose intake differences when tested with 4% sucrose in Phase II. With pups, the behavioural outcomes were more complicated. Pups given Phase I E3D/ED access to sucrose also demonstrated longer-term behavioural differences; however, the increase in consumption with E3D access emerged gradually in Phase II, and was only observed after pups were given 16% solution in Phase I. Pups needed the 16% sucrose concentration to develop the effect, while adults only needed 4% in Phase I. Overall, it seems pups are less sensitive to the ICP.

Adult 4% ED rat intake is about ½ their maximum solution intake (Adult 4% E3D rats typically double the intake of Adult 4% ED rats). This intermittent vs. continuous group pattern difference in Phase I of this experiment with adults receiving 4% is slightly less pronounced than previous work (Eikelboom & Hewitt, 2016; Senthinathan, 2012). Over each Phase I day, the Adult E3D 4% group increased their intake of solution in a fairly stable manner, suggesting that these rats had not reached their maximum, so if the Phase I was extended for a few more days it is likely that a larger difference would have developed between the intermittent and continuous adult 4% groups. In contrast, the adult 16% groups seem to maximize their solution intake (access did not influence intake), this is consistent with previous work from our lab.

I found pup ED groups seemed to maximize their solution intake at each concentration (access did not influence intake). Previous work from other labs had shown in 1-h tests of sucrose solution intake, younger rats increased intake with increasing concentrations while intake for adult rats peaked at about 4%-8% (Bertino & Wehmer, 1981; Ernits & Corbit; 1973), suggesting younger rats are tuned towards acceptance of sweeter solutions compared to adults, and this effect fades with age. More detailed description of these previous works is provided along with the implications of my findings in Chapter 5.

For pups I found 24 h volume intake of 16% solution was initially about equal to 4% intake, and pups gradually began consuming larger volumes of 4% than 16%. This 4% over 16% pattern is typical for young adult rats, so as pups grow, they develop this 4% over 16% difference typical of young adults. With young adult rats, I found they initially showed the 4% over 16% pattern, but with increasing age they seemed to shift their intake towards more equal intake of the two solutions (as noted in the “Results and Discussion: Behavioural Analysis” section above). Therefore, over adulthood, rats shifted towards accepting more calories from 16%, bringing the 4%/16% volume intake ratio closer to 1:1. Other work showed increased acceptance of very sweet solutions in much older adult rats (525-630 days of age) (Smith & Wilson, 1989).

Given younger rats are tuned towards sweeter sucrose solutions (Bertino & Wehmer, 1981), the difference between 16% sucrose and 4% might not be the same for pups and adults. Then, the shift from 16% to 4% experienced by all 16% groups is not the same because it might be considered a more negative

shift for pups, compared to adults. Because the neural activation I am exploring is in response to intake of 4% after Phase I with either solution, the adult and pup comparisons are to be interpreted with caution.

One possibility for the 16%-4% ratio difference across development involves the changing value/preference/taste of sucrose solutions. Sucrose taste thresholds (the lowest concentration at which rats will consume more solution than water) in pups and adult rats (at the ages I tested) are similar, at about 0.5% sucrose solution, and above this concentration, rats will drink more sucrose than water. Perhaps 4% is similar for pups and adults, while 16% is different because younger rats are tuned towards more intense sweetness. In other words, compared to adults, pups may be tuned towards the sweeter, but not necessarily away from the low, or mild. Then, in the neural response to 4% sucrose, we might expect some similarities between pups and adults given 4% continuously. This might also tell us something about how consumption of continuously available mild solutions are regulated by the brain; however, similarities in neural patterns of ED 4% pups and adults may not map on to behaviour directly, because the downstream effects of this activation and how it influences behaviour might be quite different in pups and adults. The influence of continuous access to mild sucrose in younger vs. older rats is re-visited in the larger discussion in Chapter 5.

Fos analysis revealed several neural structures that might be related to the Phase II ICP differences that I found with pups and adults across the experiments in Chapter 3. These include the VP, the ventral part of the lateral septum, the

parvicellular part and the dorsal cap of the PVH, and the pPVT. This work sheds light on specific structures that are uniquely engaged following a period of intermittent, or continuous access to sucrose. Future work aimed at exploring changes in individual brain structures might target these structures instead of the whole-brain analysis technique I employed. Interestingly, I did not find any differences in Fos-expression in the NA_{core} or the cingulate, which might have been expected given the work by Pecoraro and Dallman (2005), and this might be related to procedural differences.

For adult 4% ED rats, the DTT-hub was strongly activated. The DTT-hub was not found in the adult 16% or pup groups. Future work might look at how activation of this DTT-hub influences behaviour more directly. How continuous access to sucrose influences subsequent intake, and how this is mediated by age is considered in Chapter 5.

Limitations and Future Considerations

Overall, Fos counts were lower than expected. While this might be related to the repeated exposures rats had with sucrose, the overall pattern of low levels of Fos is more likely related to the Fos-IR procedures, possibly involving poor biotin amplification. If this experiment were replicated and greater amplification was obtained (and thus more Fos-expression was found in all the groups) we would still expect the same Fos-expression patterns and groups differences that I reported. It is possible that some group differences were not found due to the overall low levels of Fos-expression.

Rats were given 6 common sucrose days in Phase I. Subsequently, and coinciding with the first common sucrose day of Phase II in the previous behavioural work found in Chapter 3, rats were given a limited amount of 4% sucrose to induce Fos-expression. Considering the work in Chapter 3, at this point in the protocol, adults ED and E3D groups showed behavioural differences. Pups trained with 16% sucrose also developed behavioural differences, but at the age at which the pups were sacrificed for the Fos experiment, they do not present behavioural differences. Rather, the sucrose intake difference gradually emerges (with continued E2D exposure to 4% sucrose). Sacrificing rats at an age when the behavioural differences are present might provide different results, possibly leading to an improved interpretation of the results I have obtained.

Rats shifted from 16-4% had 4% sucrose for the first time before they were sacrificed to explore Fos-expression. Consequently, the influence of novelty on Fos-expression complicates these results. Extending this design to include a few common E2D Phase II days to avoid the influence of novelty prior to implementing the Fos protocol is suggested in future work. Alternatively, testing animals with the same solution in Phase II as they were given in Phase I might also facilitate interpretation of the results.

The neural differences I found between the intermittent and continuous groups might indicate acquired differences associated with the taste of sucrose, and these neural differences might be related to the behavioral differences that I reported in the ICP experiments in Chapter 3. Several other factors might be involved, and responsible for the behavioral differences associated with the ICP,

including differences in enzyme activity (that might result in faster or slower breakdown of sucrose) and differences in hormone activity (that might promote feeding or delay satiation). How the ICP might influence peripheral systems has not been explored.

Chapter 5: Discussion of Dissertation Findings and Research Implications

The ecological environment that surrounds an organism, including the availability of resources, can have a profound impact on behaviour, cognition, and their underlying neural processes. Once established, these changes in neural processing can have long-term influence on cognition and behaviour. Unpredictable or restrictive environments can make it difficult for an organism to predict future rewards, thus, in such environments it makes sense for an organism to learn to respond to immediate rewards. As a consequence of the uncertainty associated with infrequent or limited availability, organisms may adopt risk prone strategies, such as excessive consumption with its associated consequences (Woods, 1991).

With the ICP, the E3D access can be described as an environment of limited availability, at least when compared to groups with continuous availability. I found the environment of limited (intermittent) sucrose availability impacts sucrose intake such that adult rats increase their intake of sucrose (within satiety limits) whenever it is available. A period of E3D vs. ED access to sucrose has long-term influence on sucrose intake levels in adult rats, at least partly because intermittent rats maintain their elevated consumption and are slow to compensate by reducing their intake when resource availability improves (becomes more frequent). This behavioural phenomenon has been replicated with various palatable solutions (Celejewski, 2011; Eikelboom & Hewitt, 2016, Senthinathan, 2012; Valyear, 2014).

My MSc work with the ICP suggested the way availability influences sucrose consumption levels changes over development, so I followed up on this work and explored developmental differences in sucrose intake patterns.

Adult rats receiving intermittent vs. continuous access to 4% sucrose showed a large sucrose intake difference, and in contrast, adult rats receiving intermittent vs. continuous access to sweeter, and thus more calorically dense solutions, including 16% sucrose, did not typically show differences (Eikelboom et al. unpublished). As with adults given 16%, pup rats with intermittent vs. continuous access to 4% sucrose did not show sucrose intake differences (Senthinathan, 2012). The lack of difference in pups receiving intermittent vs. continuous access to 4% sucrose (and adults receiving more concentrated solutions) might be related to limits on consumption because of the size of the rats and maximum fluid-volume intake, caloric load of the solution, and interactions among these variables. Whether pups develop longer-term ICP differences was not clear for reasons described below (Senthinathan, 2012).

In my MSc work, rats receiving intermittent vs. continuous access to 4% sucrose from the beginning of the pup period up to adulthood, started to show a sucrose intake difference during late adolescence (Senthinathan, 2012). This sucrose intake difference emerged slowly, and gradually became larger over late adolescence, and rats showed the typical two-fold intermittent vs. continuous 4% intake difference by early adulthood. Whether the effect emerging over adolescence was solely due to the ongoing intermittent vs. continuous access during the adolescent period, or if the experience the rats had during the pup

period had some lasting impact was not clear. It is possible the difference that emerged over adolescence was (at least in part) due to the intermittent vs. continuous access the rats had as pups. Table 5.1 provides a summary of findings from all experiments in this thesis.

Findings from the Experiments

Table 5.1

Summary of Experiments.

Experiment	Rationale	Design	Age of rats	Sucrose concentration	Results
2.1	To examine how availability influences sucrose consumption across development.	Cross-sectional with 3 conditions: 4D, 20D, ED	Pups, Adolescents, Adults	4% on Day1-Day4-Day20, Day1-Day20, or ED	All ages increased consumption with 2 intermittent exposures and decreased consumption with continuous access (weight-corrected); adolescents consumed more sucrose overall than adults and pups.
3.1	To replicate 4% ICP work (ICP effect in Phase I, maintained in Phase II) with a shorter Phase I.	ICP	Adults	4% in Phase I & II	ICP effect in Phase I; maintained in Phase II.
3.2	To examine the ICP effect in pups.	ICP	Pups	4% in Phase I & II	No ICP effect in Phase I or Phase II.
3.3	To replicate 16% ICP work (no difference in Phase I, ICP effect in Phase II with 4% sucrose) with a shorter Phase I.	ICP	Adults	16% in Phase I: 4% in II	No difference in Phase I, immediate ICP effect in Phase II.
3.4	To examine whether pups could develop the ICP effect with a stronger sucrose concentration.	ICP	Pups	16% in Phase I: 4% in II	No difference in Phase I, ICP effect in Phase II gradually emerged over mid-late adolescence.
3.5	Replication of 3.4	ICP	Pups	16% in Phase I: 4% in II	As 3.4 but much less pronounced.
3.6	To replicate 3.4 and to further investigate the development of the ICP effect in pups.	ICP with gap condition	Pups	16% in Phase I: 4% in II	As 3.4 but less pronounced. Following the gap, gap groups gradually showed the ICP effect and consumed more sucrose overall than non-gap groups.
4.1	To explore neural activity associated with sucrose intake in ICP groups.	ICP Phase I only; Fos-study	Adults, Pups	4% or 16% in Phase I: 1 h 4% sucrose test	The VP, the PVH, the PVT, and the LS may be involved with the ICP-related sucrose intake differences.
4.2	To explore functional neural network activity associated with sucrose intake in ICP groups.	Network analysis of Fos data	Data from 4.1		For each sucrose concentration, consistent patterns were identified in intermittent vs continuous adult groups only.

Note. 4D= Day 1, Day 4, Day 20; 20D= Day 1, Day 20; ED = every day; E3D= every third day; E2D = every second day; ICP= Phase I E3D vs. ED, Phase II common E2D access for both groups; VP= ventral pallidum; PVH= paraventricular hypothalamus; PVT= paraventricular thalamus, LS= lateral septum.

Chapter 2

The experiment in Chapter 2 stands alone because of the unique design relative to all the other experiments, which involved some version of the ICP.

Adolescents are Different from Both Pups and Adults. Four percent solution consumption in pups, adolescents, and adults showed adolescents consume more solution per 100 g of body-weight than both pups and adults. Previous work had demonstrated similar results with adolescents and adults receiving 1% sucrose for 14 days continuously, but had not tested pups (Wilmouth & Spear, 2009).

The experiment in Chapter 2 also showed the pups, adolescents, and adults that received sucrose on Day 1, followed by a gap without sucrose and sucrose again on Day 4 (4D group), increased their intake while age-matched rats with continuous access decreased their intake. From Day 1 to Day 4, pup intermittent groups increased their sucrose intake while continuous groups decreased their intake (per 100 g of body-weight). This decrease in the continuous groups is at least in part due to body-weight increase. A closer look at the ED groups in Chapter 2 shows that from Day 1 to Day 4, pups with continuous access increased their volume intake of solution, while adolescents are slightly lower on Day 4 than Day 1, and adult groups are clearly lower on Day 4 than Day 1. Even though the pup continuous group clearly increased volume intake from Day 1 to Day 4, their sucrose intake per 100 g of body-weight decreased because of body-weight gain. I found a similar result with pups, adolescents, and adults that received sucrose on Day 1, followed by a gap

without sucrose and sucrose again on Day 20 (20D group), with intermittent groups increasing intake while continuous groups decreased intake.

The experience for the 4D group (rats that received 4% solution on Day 1, Day 4, and Day 20) from the experiment in Chapter 2 is like the experience for the intermittent E3D group in the ICP on their first and second sucrose day (both receive sucrose on Day 1 and Day 4). Additionally, both the 4D group from Chapter 2 and intermittent groups in all subsequent ICP experiments, are compared to a group of rats receiving sucrose continuously. The lack of an age difference between the pups and adults in the D4 groups (Chapter 2) might seem surprising because in all other cases I report pups do not develop differences with intermittent vs. continuous access to 4% sucrose. However, the comparison in Chapter 2 involves Day 1 and Day 4, and consumption per 100 g of body-weight, whereas with all ICP experiments, I analyzed the full Phase I (the comparison involves Day 1, 4, 7, 10, 13, and 16), and volume of solution consumed. The seemingly discrepant findings may be related to the differences in data analysis and statistical procedures between the experiment in Chapter 2 and all other work in this dissertation as well as my previous work in pups (Senthinathan, 2012). Close inspection of the experiments with pups given 4% (Chapter 2, Chapter 3 Exp. 2, and my MSc work) revealed that on Day 4, intake by pups with E3D access to 4% sucrose is slightly above pups with ED access. My MSc work showed this very slight difference is stable across the pup period, and it only becomes larger after the pup period. Importantly, the ICP experiments with pups (described below) suggest pups given intermittent vs. continuous

access to 4% do not develop longer-term differences. Thus, the slight sucrose intake difference with pups receiving intermittent vs. continuous access to 4% does not seem to have a lasting impact.

Chronic Mild Sucrose Exposure over Development. Rats given continuous access to 4% sucrose from the beginning of the pup, adolescent, and adult periods, respectively, did not differ in body-weight gain and consumed similar levels of sucrose as adults. The age and developmental stage at which the mildly sweet solution becomes chronically available had no statistically significant influence on sucrose intake. However, closer examination of the data suggests there might be a difference between the rats that began with sucrose as adolescents, and the rats that began with sucrose as pups or adults. As same-aged adults, the adolescent group maintained a slightly higher consumption over the final 14 days of the experiment compared to the other two groups. A replication with more rats in each group might find the adolescent group maintains higher intake levels over the other groups in this period.

Chapters 3-4: ICP Experiments

Most of my experiments only involved pups, adults, or rats at both developmental periods, tested in some version of the ICP. The ICP experiments were designed to mirror sucrose experience in adults and pups. Thus, the duration of Phase I had to be limited to the duration of the pup period, which is only about 16 days.

In all ICP experiments, behavioural differences in Phase II demonstrate longer-term changes induced by the pattern of availability (intermittent vs.

continuous access) between the groups in Phase I. Phase II was always a common schedule for both groups, with alternate-day access to 4% sucrose. The ICP experiments involving pups continued into their adolescent period and Phase II was during adolescence, but importantly, any group differences are solely attributed to availability of sucrose during the pup period as the beginning of Phase II coincided with the end of the pup period.

Phase I.

Adults. With 4% sucrose, adult rats receiving the solution intermittently vs. continuously quickly showed a large solution intake difference. Adult 4% ED rat intake is about ½ their maximum solution intake (Adult 4% E3D rats typically double intake of Adult 4% ED rats). This intermittent vs. continuous group pattern difference in Phase I with adults receiving 4% is slightly less pronounced in Chapter 3 (Experiment 1) when compared to the experiment in Chapter 4, as well as previous work from our lab. In the experiment in Chapter 3, the continuous rats consumed more than what we typically find, resulting in a smaller overall intermittent vs. continuous difference (continuous rats were consuming about 150 g at the end of Phase I while intermittent rats consumed about 225 g). In Chapter 4, the intermittent vs. continuous 4% sucrose intake difference (about 2:1) is similar to what we have found previously (Eikelboom & Hewitt, 2016; Senthinathan, 2012). In the experiment in Chapter 4, over each Phase I day, the Adult E3D group consistently increased intake, suggesting that these rats had not reached their maximum, so if the Phase I was extended for a few more days it is possible that an even larger difference would have developed between adult

groups with 4% sucrose (continuous rats were consuming about 100 g at the end of Phase I while intermittent rats consumed about 200 g).

With 16% sucrose, adult rats consumed similar levels in Phase I (Chapter 3 Exp 3; Chapter 4 Exp 1). Adult 16% groups seem to maximize their solution intake (access did not increase intake), and the lack of difference with 16% is likely due to the limiting effect of satiety on intake in intermittent rats, such that they are not able to increase intake above the amount consumed by continuous rats. In adults, the intermittent vs. continuous Phase-I difference with 4%, and lack of this difference with 16%, replicates previous work (Eikelboom et al., unpublished).

With the ICP, adult rats do not show a difference in Phase I with 16%, but they clearly show a large difference in Phase I with 4%. Similarly, with the M-W-F protocol, adult rats receiving 1.5 h access with 3.2%, 10%, or 32% sucrose intermittently M-W-F, vs. continuously, show a significant intake difference at the mild and moderate concentration, but do not show differences with the highest (32%) solution (Wojnicki et al., 2007). With higher sucrose concentrations we are less likely to observe behavioural changes even though underlying changes may have developed, because rats are more motivated to consume sweeter solutions than less sweet solutions, and because of satiety-effects that prevent intermittent rats from increasing their intake.

Pups. For pups, the findings in Phase I are similar with 4% solution, and 16% solution (Chapter 3 Exp. 2, 4-6; Chapter 4 Exp. 1). Pups did not show an intermittent vs. continuous difference during Phase I with either solution.

Developmental mechanisms may render pups less sensitive to environmental influences on feeding. For example, pups may be set to consume maximally (Spear, 2000), which may help to explain why I did not find Phase I intake differences in pups. As with adults receiving 16% described above, the lack of a difference in Phase I, both with pups receiving 4%, as well as 16%, might be related to satiety.

Pups and adults tested together with 4% and 16% in Phase I (Chapter 4)

In Chapter 4 I tested pups and adults with a version of the ICP designed particularly to explore neural differences related to the consumption patterns we find with the protocol. Phase I was identical to all the previous ICP experiments, but this experiment tested rats at both ages, and with both solutions, thus permitting direct comparison between intake of the two solutions at each age.

Adult ED groups consumed about 1.4 times more 4% solution than 16% solution on Day 1, and this 4%>16% consumption difference became smaller across Phase I. Previous work had reported sucrose acceptability for adult rats over 24 h is similar with both solutions (Smith & Wilson, 1989). This is in accord with what I found by the end of Phase I as intake levels for the two solutions got closer to 1:1. In pups, initial intake of the two solutions was similar and a difference gradually emerged with pups consuming more 4% than 16% solution. To our knowledge, this age difference (increasing ratio in pups compared to decreasing ratio in adults) between pups and adults has not been shown previously. Previous studies exploring sucrose intake in rats have typically focused on adult rats (Sclafani & Nissenbaum, 1987; Smith & Sclafani, 2002;

Spector & Smith, 1984; Smith & Wilson, 1989; Young, 1948). Work that has tested rats across the lifespan has not explored early developmental aspects, and instead grouped pups and adolescents with young adult rats to compare them to older adult rats (Smith & Wilson, 1989).

Many consumption related behaviours typically observed in older rats show a developmental trajectory (Cortright, Chandler, Lemon, DiCarlo, 1997; Dalton-Jez, 2006; Klump et al., 2011; Mastroianni, 2013), which is similar to the 4% > 16% intake difference I found with pups. Since the difference in 4% vs. 16% solution intake was not evident in very young pups, and only developed gradually, this might suggest the earlier lack of difference might be related to limits on fluid-volume, taste sensitivity, or preference for the two solutions in pups.

Short Phase I: Longer-Term Difference in Phase II. In all of my Chapter 3 ICP experiments in this dissertation with adults and pups, after Phase I, the intermittent and continuous groups where shifted to alternate-day access to 4% sucrose in a Phase II, and sucrose intake in Phase II was analyzed to assess if Phase I had a lasting influence on sucrose intake. Previous work with the ICP had almost exclusively focused on testing adult rats, and typically had a longer Phase I (typically ~10-13 common sucrose days) (Eikelboom & Hewitt 2016; Senthinathan, 2012; Senthinathan & Eikelboom, 2011; 2012; 2013; 2014) compared to the shorter Phase I in my dissertation (6 common sucrose days).

Adults. With a relatively short Phase I version of the ICP, this work replicates the patterns we usually find with longer Phase I studies. The Adult 4%

E3D rats developed and showed a pattern of increased sucrose intake compared to the Adult 4% ED rats in Phase I, and this pattern continued in Phase II (but with a nonsignificant trend). The overall intake difference between the intermittent vs. continuous groups immediately reduced in Phase II but the elevation in the intermittent rats was maintained, while the continuous rats rapidly increased their intake (Ch. 3, Exp. 1). Similar results were demonstrated by Eikelboom and Hewitt (2016).

Receiving the sweeter 16% solution, adult rats developed an unexpressed difference (i.e. the difference developed in Phase I, but was not evident, or was not expressed) (Ch. 3, Exp. 2). In Phase I with 16%, adult intermittent and continuous groups consumed similar amounts of solution, however a clear difference immediately showed in Phase II when the sucrose concentration rats were receiving was lowered from 16-4%. Previous ICP work with a longer Phase I showed this 16%-4% pattern and ICP effect in adult rats (Eikelboom et al. unpublished).

Pups. The only previous work in pups with the ICP that we are aware of is my MSc work. In my MSc work, I tested rats from the pup to the adult period with a 4% solution. Rats were shifted to Phase II as adults. In Phase I, pups did not present the sucrose intake difference, and the intermittent vs. continuous effect we typically find with older rats given 4% sucrose gradually developed across the adolescent period. It seemed that the influence of availability on behavioural patterns in pups was different.

A major focus of my experiments in this dissertation was exploration of early life access (i.e. the pup period in rats) to sucrose to assess whether patterns of availability experienced during the pup period can promote longer-term behavioural differences.

With 4% sucrose, the rats did not show a difference in Phase I as pups, with intermittent vs. continuous access, and no difference emerged during adolescence in Phase II (when rats were maintained on E2D access with 4%) (Chapter 3, Exp. 2), so it seems with 4% the ICP does not have a lasting influence on pups. In my MSc work, I found the same Phase I result during the pup period, but with a longer Phase I (continued after the pup period) a difference emerged over the adolescent period (Senthinathan, 2012). The emergence of the difference over adolescence in my previous work was likely due to the rats E3D vs. ED experience during the adolescent period, otherwise, we might expect a difference in Phase II in the second experiment in Chapter 3.

Pups developed a late emerging ICP difference with the more intense solution (16% sucrose), thus pups are not invulnerable to ICP effects. It has been shown that pups choose to consume sweeter solutions than adults (Bertino & Wehmer, 1981). The changing hedonic value of sucrose across age-development likely contributes to why pups don't develop a Phase II difference when given the lower 4% (compared to 16% sucrose) in Phase I, while adults develop a Phase II difference when given 4% in Phase I. Perhaps the reason pups don't develop the ICP difference with 4% is because for pups, the reward value of 4% is too low. Pups may be just as sensitive to reward scarcity or

uncertainty as adults, and the difference I found with pups and adults might be related to perceptual differences in the rewarding value of each solution across age. With weak (less rewarding) solutions, adult rats do not develop the ICP difference (discussed below in “Sugar as a Reward”).

Interestingly, when the effect developed in pups, the difference was not observed until mid-adolescence (Ch. 3 Exp. 4-6). Developmental mechanisms can prevent the expression of underlying problematic behaviours, possibly making underlying changes less likely to show (Klump et al., 2011). To follow up on this, I did the gap experiment in pups, described below.

The Gap Experiment. All experiments in Chapter 3 involved groups of rats given intermittent vs. continuous access to sucrose (4% or 16% in Phase I, and always 4% in Phase II). The final experiment in Chapter 3 tested pups given intermittent vs. continuous access to 16% sucrose and had an additional gap condition (ten-day gap between Phase I and Phase II), creating 4 groups. Perhaps the most remarkable result I found across experiments was that following the shift from Phase I to Phase II and after the long gap without sucrose, these pup gap groups showed the same pattern as the more conventional groups. This underscores that the influence of reward-availability in pups can be very robust and long-lasting. Additionally in comparison to the non-gap groups, the sucrose intake by the gap groups was shifted upwards, demonstrating the sucrose deprivation effect (a known effect described by increased intake of sucrose following a prolonged gap period (Gandelman & Trowill, 1969; Pinel & Rovner, 1976; Sinclair & Senter, 1967; 1968) and

discussed in Chapter 3) and the longer-term intermittent vs. continuous consumption difference (Eikelboom & Hewitt, 2016; Rhen & Boakes, 2019; Senthinathan, 2012; Wayner et al.,1972; Wise, 1973) act independently.

The gap experiment (Chapter 3 Experiment 6) showed that following Phase I and the gap without sucrose, a difference between the gap groups gradually emerged, while the difference in the groups without the gap was much larger. After the gap, a few days with sucrose were needed to show the "hidden" difference in the gap groups. Ueji and Yamamoto (2014) showed young adult rats (age 56 days) given 15-minute two bottle choice tests with 2% sucrose, and 30% sucrose simultaneously on alternating days consumed similar levels initially, but quickly (by 60 days, the 3rd exposure) showed greater intake of 30% over 2%. This lab also showed that in 21-day pups, 15-minute two bottle choice tests with 2 and 30% sucrose presented every other day, or every two weeks (days 21, 35, 49, 63, 77), or every four weeks (days 21, 49, 77) showed that in the every other day group a difference very gradually emerged with pups beginning to consume more of the higher solution than the lower solution by 39 days (the 9th exposure), the beginning of the adolescent period (Ueji & Yamamoto, 2014). In the other groups (every two-week group and every four-week group), the rats took longer, until 77 days to show the 30% > 2% effect (Ueji & Yamamoto, 2014). Thus, Ueji and Yamamoto (2014) showed early experience with a solution shapes how it is consumed, and some age-dependent differences might also require experience to become evident, which is exactly what I found with the +Gap groups in the gap experiment. In the gap experiment, with the ICP and

pups given 16%, the intermittent vs. continuous access to the solution had a lasting influence on consumption, but the effect only emerged later in life. For +Gap groups, the effect took longer to emerge than for the other intermittent vs. continuous groups, suggesting that more experience with sucrose after Phase I was needed for the difference to emerge. Thus, the longer-term (Phase II) ICP effect can develop in pups, but is latent and only emerges across adolescence, and seems to require sucrose experience in adolescence for the effect to emerge.

Behavioural patterns can develop during the pup period and remain hidden until the adolescent period or later in life. Several classic works have shown that learning can occur without observable change (Amsel, 1994; Ross, 1964). This idea was introduced almost a century ago (Blodgett, 1929), and is concisely summed by the traditional aphorism “absence of behaviour is not evidence of absence”. My work revealed an unsuspected protracted effect of sucrose availability in pups on behaviour, and this was particularly striking in the “gap experiment”.

ICP Phase II: 1 h Sucrose Test. In Chapter 4, after a 16-day Phase I with intermittent vs. continuous access to 4% or 16% with pup and adults, and Day 17 without sucrose, all rats were given 1 h to consume a limited amount of 4% sucrose in a Phase II in order to induce sucrose related Fos-expression in the various groups.

Overall, pups consumed more 4% sucrose than adults during the 1 h sucrose test. This 1 h sucrose intake difference between younger and older rats,

complimented by other data showing adults will consume greater volume of 4% than younger rats at the ages I tested (clearly shown in Chapter 2), suggests that the way animals consume sucrose in 1 h tests might be quite different from consumption in longer tests. This is consistent with Adam Celejewski's work from Eikelboom's lab, as well as others (Monk et al., 2014).

Neural Differences. Regulatory feeding to maintain body-weight, and hedonic feeding in the absence of need are regulated by separate, and overlapping systems (Castro et al., 2015; Rossi & Stuber, 2018). I quantified Fos-expression in several reward-related brain areas to explore sucrose intake-related neural activity in structures that have been implicated in the brain's reward system (Castro & Berridge, 2014; Ho & Berridge, 2013).

The work in Chapter 4 represents a preliminary attempt to explore neural differences related to sugar consumption in rats with varying sucrose experience. Via quantification of Fos-IR associated with the sucrose test, I identified several neural structures that might be related to the longer term-behavioural patterns we find with the ICP. These include the ventral pallidum (VP), the ventral part of the lateral septum (vLS), the parvicellular part and the dorsal cap of the paraventricular hypothalamus (pPVH; dPVH), and the posterior part of the paraventricular thalamaus (pPVT). All of these structures have been previously linked to palatable food intake or sucrose consumption (Covelo et al., 2014; Pecoraro & Dallman, 2005; Castro & Berridge, 2014). The involvement of each of these structures in feeding and sugar consumption is briefly described.

The VP is known to be particularly involved in palatable food consumption, with previous work showing that increasing or decreasing activity in the VP by drug administration has a direct impact on intake of palatable foods (Covelo et al., 2014). The LS is also intimately involved in palatable food intake (Mitra et al. 2014). A circuit connecting the hippocampus with the LS is known to regulate feeding (Sweeney & Yang, 2015), and other work suggests that the LS is important for the development of sucrose overeating (Mitra et al. 2014). The PVH is also important for regulatory feeding, including detecting glucose levels or "glucose sensing" and ensuring adequate glucose levels are maintained for brain function (Routh et al., 2014). This structure is also sensitive to palatable food, with sweetened condensed milk intake selectively increasing Fos-expression in the PVH (Hume et al., 2017). Therefore, the VP, LS, and PVH are likely involved in the intermittent vs. continuous differences in our experiments, and the Fos findings might relate to some of the behavioural differences we see in the ICP.

Other work has shown chronic exposure to a high-sugar diet is associated with reduced Fos-expression in the PVH following intake of either high sugar or a bland diet compared to sugar naïve rats (Mitra et al., 2011). The PVH may be involved in regulating intake based on availability.

The role of the PVT in feeding and reward related brain circuitry was described recently (Kirouac, 2015). In relation to feeding, the pPVT receives signals from first-order taste centers in the hindbrain and may be involved in guiding behaviour associated with the rewarding and aversive properties of food (Igelstrom et al., 2010; Kirouac, 2015; Yamamoto et al., 1995; Yasoshima et al.,

2007). Thus, the pPVT is likely involved in determining the emotion valence for various sucrose solutions.

Taking a systems approach to explore the data, I applied complex network analysis to the c-Fos data set (Chp. 4, Exp. 2). Network analysis identified the DTT as a particularly important “hub-like” structure in a functional neural network (involving the dorsal part of the lateral septum, the ventral part of the PVH, the cingulate gyrus, the lateral part of the substantia nigra, the ventral part of the BNST, the supraoptic nucleus, the piriform area, and the locus coeruleus) in the Adult ED 4% group. The DTT-hub was not found in adults that had continuous access to 16% sucrose, possibly because they had been trained with the sweeter solution, but given 4% sucrose to induce Fos-expression. The DTT-hub was not found in the pup groups. Since the DTT-hub was only found in the adult ED 4% group (adult group that does not develop increased sucrose intake), and not found in the pups, the DTT-hub might be particularly important for regulating sucrose intake in adult rats when it is regularly available.

The interaction between availability and reward might change with development. Results from the network analysis showed some parallels with our behavioural findings, as well as some inconsistencies, which are discussed in Chapter 4. Overall, this preliminary ICP-Fos-Network study seems promising and warrants further investigation.

Implications for Theory, Research, and Further Discussion

Sucrose Sensitivity and Age

The preference for 1% sucrose solution over water can be used to measure sucrose sensitivity and anhedonia (Willner et al., 1987). With the ICP, adult rats receiving intermittent vs. continuous access to 1% sucrose do not show differences in Phase I, or in Phase II (with common E2D access) (Eikelboom et al., unpublished). Since adult rats choose to consume 1% over water (Wilmouth & Spear, 2009), the lack of an intermittent vs. continuous difference with this low solution is not because of lack of sensitivity or preference for 1% over water. It might be related to the perceived rewarding value of sucrose.

Sucrose sensitivity changes over development. Adult rats receiving a choice between 1% sucrose and water increase their intake of sucrose over water and while adolescents show a similar pattern, the increase in solution intake over water is larger compared to in adults (Wilmouth & Spear, 2009). We are not aware of studies that compared pups and adolescents so it is not clear if 1) rats show an age-related decline in sucrose sensitivity from the pup period onwards, 2) sucrose sensitivity peaks during adolescence (and pups and adults might either be the same or different), or 3) the sucrose sensitivity is maintained in younger rats and declines in adulthood. Given several studies have shown adolescents are more sensitive to sucrose than adults (Bertino & Wehmer, 1981; Naneix et al., 2016; Wilmouth & Spear, 2009), and that I found adolescent rats consumed more sucrose (adjusted for body-weight) than pups and older rats, overall the work seems to support suggestion #2, sucrose sensitivity peaks

during adolescence. I found no initial differences in consumption of 4% and 16% in pups (Chapter 4, Experiment 1). Perhaps the intake difference between rats receiving 4% and 16% that gradually emerged close to the end of the pup period is related to a gradually emerging age-related peak in sucrose sensitivity.

Young rats receiving continuous access to 5% sucrose from 30-46 days in age showed reduced sucrose sensitivity (reduced preference for 1% sucrose over water, increased anhedonia; this reduced sucrose sensitivity effect was not found in adults, described below), and complimentary changes in other emotional behaviours (including decreased motivation for saccharin and increased immobility in the forced swim tests) as adults, when compared to age-matched sugar naïve rats. Following the same procedure (15-day access to 5% sucrose) with adult rats showed no difference in sucrose sensitivity from controls, but changes in some of the other emotional behaviours were evident (Gueye et al., 2018). Thus, sucrose experience can have long-term influence on sucrose sensitivity, an effect mediated by age. Gueye and colleagues (2018) reported that overall, in adult rats, continuous access to 5% sucrose may also have a long-lasting impact on emotional and reward related behaviours. Heightened sensitivity to sucrose during adolescence might make rats at this developmental stage more sensitive to changes caused by sugar consumption than adult rats. Adolescent rats are both more sensitive to sucrose and to the changes associated with continuous sucrose intake compared to adult rats. Reduced sucrose sensitivity is associated with reduced intake of 5% sucrose (Naneix et al., 2016). So, increased sucrose sensitivity might increase intake of 5% (and

likely, 4%) sucrose. My work in Chapter 2 might suggest that continuous access to a mild solution during either the pup period (22-37 days) or adulthood (56-76 days) may not result in increased sucrose sensitivity, and that continuous access to sucrose during adolescence results in increased sucrose sensitivity. This would then suggest that the work by the Cador group showing age-effects in younger rats given continuous access to mild solution over parts of the pup and adolescence periods (30-46 days) compared to older rats is due the continued access to sucrose over adolescence (37—46 days).

I stated (above), that overall my work and previous literature seems to support suggestion #2, “sucrose sensitivity peaks during adolescence (and pups and adults might be either the same from each other, or different)”. Rats with less sucrose sensitivity tend to consume more solution at very strong concentrations (vs. rats with greater sucrose sensitivity). On the other hand, rats with greater sucrose sensitivity are more likely to consume more of a very weak solution (vs. rats with less sucrose sensitivity) (Wilmouth & Spear, 2009). Even if pups are more sensitive to mild solution compared to adults, it is unlikely that we would find any ICP effects with lower concentrations given pups do not develop the ICP effect with 4%.

Age Influences Learning Processes

The persistent differences we see in Phase II of the ICP must be underpinned by some form of learning. Age-related differences in learning processes might contribute to the difference I found with pups and adults. The partial-reinforcement-extinction-effect (PREE) as well as several related effects

gradually emerge across development (Amsel, 1992; Burdette et al., 1976).The PREE describes a phenomena whereby intermittent reinforcement produces much more robust responding compared to continuous reinforcement, and is significantly more resistant to extinction procedures during which rats are no longer rewarded for making the goal response (Amsel, 1992). The PREE shows that rats trained with intermittent schedules vs. continuous schedules develop persistent behavioural differences, which is like what we find with the ICP. The PREE emerges preweaning, so pups and adults both show the effect. Pups trained on reinforcement schedules show greater resistance to extinction than adults and the effect is particularly evident in partial vs. continuous reinforcement schedules; the effect is more pronounced in pups (Burdette et al., 1976). I found the opposite age-pattern such that adults that received 16% or 4% sucrose intermittently vs. continuously developed a difference while pups only developed the difference with the stronger solution, so pups are less sensitive to developing differences with the ICP. Thus, pups are more sensitive to the PREE than adults, but less sensitive to the ICP than adult rats.

In another well-known paradoxical appetitive learning effect, for rats receiving continuous rewards during training, greater reward is associated with faster extinction (North & Stimmel, 1960). This effect has been called the overtraining-extinction-effect (OEE) and is evident across the rat lifespan but is more pronounced in older rats and less pronounced in pups (Burdette et al., 1976). Thus, the greater reward-continuous reinforcement-age effect seems to mirror the developmental trajectory of the effect I found with the ICP. Like the ICP

effect, pups are less sensitive, and as rats get older, they become more sensitive. A closer look at what we typically find with the ICP might be informative.

With 4%, the ICP did not influence intake in younger rats, but I found that this lack of sensitivity to the ICP could be overcome with stronger (16%) solution. Adult E3D rats given 4% sucrose quickly increase intake over days while ED rats typical reduce intake from initial levels, consequently, we find about a two-fold difference in Phase I (E3D vs. ED to 4% in adults). When shifted to a common E2D Phase II, E3D rats continue to consume similar amounts of 4% and ED rats increase their intake but continue to consume less than E3D rats. In Phase II, why would E3D-E2D rats consume more than ED-E2D? One possibility is that the E3D access results in robust longer-term change, which is supported by my previous work showing rats shifted from E3D-ED continued to consume elevated levels of sucrose, gradually reducing intake over a month (Senthinathan, 2012). Perhaps the standard ICP (E3D vs. ED followed by E2D for both groups) is ideal for highlighting the difference between the groups. In my MSc I found that rats shifted ED-E3D don't seem to show any residual effects of Phase I, but rats shifted E3D-ED show a long-lasting sucrose effect of elevated intake. Other work from our lab has shown rats that only experience E2D (i.e. E2D-E2D) consume similar levels as E3D-E2D rats in both phases, which might suggest the difference between E3D and E2D for rats is not a very meaningful change, otherwise we might expect consumption to change with the access shift. In contrast, the ED to E3D shift might be more meaningful to rats since this access

shift results in increased sucrose intake. Eikelboom and Hewitt (2016) showed in Phase II of the ICP that E2D-E2D rats appear to consume more than ED-E2D. This result shows some change and reduced sucrose intake in the continuous (ED) group (Eikelboom & Hewitt, 2016). With the ICP, it is likely that both the continuous and intermittent experience in Phase I has some influence on sucrose intake patterns. Thus, part of the Phase II difference is related to continuous access in Phase I, which shifts consumption down so the effect (intake difference) is large. In my work, perhaps the reason pups given 4% do not show the effect in Phase II is because the continuous access does not have the same influence on pups and adults. In other words, the continuous access to 4% sucrose in adults had some lasting influence of shifting 4% intake down, while in pups it might not. This argument seems to be congruent with pups being less sensitive to continuous reinforcement than adults. Since the ICP effect operates at both ages, and pups are simply less sensitive, the age-effect can be overcome with a stronger solution.

Reinforcement schedules have been shown to influence more complex learned behaviours, including spatial learning in the Morris water-maze task (Prados et al., 2008). Rats trained to swim to a particular platform (and escape drowning), by following various landmarks with intermittent vs. continuous reinforcement schedules show the typical PREE, as rats that were continually reinforced during training show less resistance to extinguishing this behaviour during extinction trials. This work demonstrated that the long-known PREE was not only related to instrumental conditioning as described above, but that it

applies to spatial learning, and furthermore in a second experiment (described next), that similar principles govern associative learning. Groups of rats were trained to find a platform with a single cue that highlighted the location on continuous vs. partial reinforcement schedules. Next, these rats were trained on a new task with various new landmarks, and importantly, with the same reinforcer as the previous task. Measured by faster escape times, rats from the partial reinforcement group from the first task performed better than the continuous reinforcement group on the second task, and the authors noted this might be related to a difference in salience of the reinforcer between the groups.

Continuous reinforcement, or continuous availability, might reduce the salience of a reinforcer. Contrastingly, intermittent reinforcement seems to maintain the salience of a reinforcer (Prados et al., 2008). With the ICP we find intermittent access seems to increase consumption of a rewarding solution, which seems different than just maintaining the salience. The type of change we find with the ICP seems more in-line with the idea that some value has been added to the intrinsic rewarding value of the reinforcer. Related to this, Celejewski's (2020) ongoing work on the microstructure of ingestive behaviour in rats is finding evidence that adult intermittent rats come to value sucrose more than rats with continuous access. It seems that intermittent access might increase the significance of an otherwise less significant item. Eikelboom's lab is currently exploring these possibilities.

Sugar as a Reward

Sugar is innately rewarding and liked, and the rewarding value of sucrose changes developmentally (Bertino & Wehmer; 1981; Ernits & Corbit, 1973; Ueji & Yamamoto, 2014). Adult rats given sucrose for 1 h show maximum volume intake with solutions between 3-6% and less intake for lower and higher concentrations (Ernits & Corbit, 1973). Younger rats show increased intake with increasing concentration (at least from about 1% to 17%), transition towards less sweet solutions around 56 days (intake peaks at 10% and is stable or lower with 17%), and develop the previously reported adult pattern by about 84 days (peaked intake at about 5%) (Bertino & Wehmer, 1981). I found that adult rats consume similar amounts of 4% and 16% solution, but pups consumed more of the 4% than the 16% solution (Chapter 4). Thus, the prior literature and my current findings suggest that the rewarding value of sucrose changes developmentally. Given I found that adult rats develop the Phase II ICP effect with both 4% and 16% while pups only develop differences with 16%, age-related differences in the rewarding value of sucrose might contribute to why pups do not develop the ICP effect with the 4% solution.

If pups are less sensitive to ecological influences on consumption then we might expect pups would require a stronger solution than adults to develop a difference with the ICP. Perhaps 4% solution is similar for pups and adults, but 16% is different, with pups valuing the higher solution more than adults. Pups might give more attention to the stronger solution, and so pups develop the difference with 16% sucrose, but not the weaker 4% solution (while adults are

more sensitive to ecological influences on consumption so they develop the difference with both solutions). A follow up study might test pups with various solutions between 4-16% to find the concentration required for pups the develop the difference. It is not likely important to test solutions lower than 4% in pups because a difference is not predicted as discussed in the “Sucrose Sensitivity and Age” section above.

Reward related consumption behaviour is often discussed in three separate components: hedonics (liking), incentive motivation (wanting), and reinforcement (learning). These components of reward-related behaviour seem to be regulated by separate neural substrates (Berridge et al., 2009; Salamone & Correa, 2012). For example, dopamine in the nucleus accumbens (NAc) plays a predominant role in reward-related learning, and “wanting” behaviours, while opioid and GABA systems plays a greater role in “liking” behaviours. Continuous access to sucrose can affect sucrose “liking” (Wiss et al., 2018). Continuous access to 5% sucrose from 30-46 days of age (i.e. beginning during the pup phase and across early adolescence) caused reduced consumption of sweet solutions at 70 days of age, as well as reduced hedonic reactivity measured by orofacial reactions to intraoral infusion of sweet solutions and was associated with reduced neural activity in the NAc compared to sucrose naive rats (Naneix et al., 2016). Wiss and colleagues (2018) suggests these types of changes are related to alterations in the brains “liking” system. The changes described above are in response to a period of continuous access. Perhaps the differences we find in Phase II of the ICP are related to “less liking” in continuous rats while

intermittent rats do not develop “less liking”. Alternatively, in my MSc I found that a long period (36 days) of continuous access to 4% followed by a shift to E3D access did not have any residual influence on intake (compared to sucrose naïve rats given E3D access). This seems to contradict the suggestion that rats develop “less liking” following a period of continuous access. It is important to note however, that my work was done with adult rats and prior work by the Cador group (Naneix et al., 2016; Wiss et al., 2018) was done across parts of the pup and adolescent periods.

Sugar is innately rewarding and liked; however, sucrose liking changes developmentally (Bertino & Wehmer, 1981). Early in development rats prefer more intense sucrose (i.e. more calorically dense) concentrations, and the intensity of preferred solutions declines with age[6]. This increased preference for sweets in younger organisms might be an evolutionary protective mechanism that promotes increased consumption of calorically dense foods during this particularly sensitive period of brain development. Brain mechanisms sensitive to the availability of various high calorie food sources, including sucrose, were likely adaptive in the evolutionary context of limited food availability.

The work by Naneix and colleagues (2016) showed continuous exposure to 5% sucrose early in development and adolescence had a lasting impact on sucrose consumption such that rats seemed to learn to like sucrose less than

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[6] Much later in adulthood, taste sensitivity declines, along with increased in preference for very sweet solutions related to the decline in taste sensitivity in older adult rats (Inui-Yamamoto et al. 2017) and complimentary increased acceptance of very sweet solutions (Smith & Wilson, 1989).

those that did not have experience with sucrose. Other work with rats given 5% sucrose at the same age (30-46 days) showed similar findings (Vendruscolo et al., 2010). Naneix et al. (2016) and Vendruscolo et al. (2010) both involved rats receiving 5% sucrose continuously, over the (late 30-38 day) pup period, and (early 39-46 day) adolescent period, so the sucrose exposure overlapped both periods. The longer-term effects of continuous 5% sucrose exposure in these studies might be due to the experience rats had as adolescents. This unknown can be resolved by testing rats strictly during the pup period (e.g. 22-37 day) with the 5% sucrose paradigm used by the Cador group. Adolescents may be most sensitive to developing longer-term behavioural changes because reward and motivation related areas in the brain undergo significant development and reorganization during this period (Spear, 2000; Simon & Moghaddam, 2015; Zoratto et al., 2018), which could explain why rats develop such longer-term differences with a short period of continuous exposure to sucrose in adolescence.

Nutrition sources that are irregularly available might develop increased value to promote intake of these less available options that might have important nutritive benefits. Adolescence is the period during which rats typically venture away from the nest and become responsible for their own food (Thiels et al., 1990) so sensitivity to availability of nutrient sources is particularly functional during this period (and not in pups) as it could shape an organism to be successful in any environment. One possibility is that via some learning process that is more pronounced in older rats and less pronounced in pups, availability

impacts the hedonic value, or liking of sucrose. As such, unlimited availability might reduce sucrose consumption and limited availability might maintain or increase sucrose consumption. Some work suggests when reward intake increases over time, or becomes excessive, it is reflective of addiction-like behaviour (Ahmed & Koob, 1998; Edwards & Koob, 2013).

Sugar as an Addiction

Addiction is a human phenomenon that has been studied extensively in rats (Kuhn et al., 2019; Lynch, 2018; Spanagel, 2017). Like the ICP effect, addiction in humans has a developmental trajectory. It is well known that addiction is influenced by age, and most developmental work on this topic has focused on adolescents (Bava & Tapert, 2010; Crews et al., 2019; Gladwin et al., 2011; Hammond et al., 2014; Jordan & Andersen, 2017; Potenza, 2013; Shramm-Sapyta et al., 2007; Winters & Arria, 2011). Human and rat work both suggest adolescents might be most sensitive to developing addictions compared to other developmental periods. Work in humans suggests childhood is associated with reduced susceptibility to addiction-like changes (Jordan & Anderson, 2017) which seems parallel with my results in pups. Research on humans, and the relevance of my work to human behaviour is discussed below, under “Relevance of this rat work to humans”.

Including the ICP, at least three related behavioural protocols consistently show animals escalate their intake of a palatable food or sugar when it is provided intermittently (12h-12h, M-W-F, ICP). With the ICP we typically focus on daily consumption while other protocols have greater focus on binge-behaviour

over a shorter initial period. All of the protocols show escalation of intake or increased intake over time. Escalation of intake has been suggested to be a core part of the addiction process (Ahmed & Koob, 1998). For an extensive review of studies testing if addiction-like qualities including bingeing or escalation, withdrawal, craving, and cross-sensitization to other rewarding substances (akin to a “gate-way” effect among substances) are imparted by intermittent access to a palatable food source, see Avena and colleagues (2008) and Corwin and colleagues (2011).

The various rodent models have been used to explore addiction-like food consumption and overall the work shows that the influence of intermittent availability is critical (Avena et al., 2008; Corwin & Babbs, 2012). Invariably, in these models of increased or addiction-like food consumption, rats with more frequent access do not develop addiction-like patterns of consumption or related neural changes, and rats with intermittent access develop the addiction-like changes.

Sugar addiction is not recognized in the current DSM-5, but the term is popularly used to describe an apparent inability to control intake of sweet foods. Some evidence suggests sucrose consumption behaviour can resemble drug addiction (Avena et al., 2008). Addiction often involves the following three steps 1) escalation of intake, 2) withdrawal, when the addictive stimulus is not available, and 3) after a period of abstinence, relapse when the addictive stimulus becomes available, which all have been demonstrated with sugar. Researchers have shown addiction-like behaviours in rats including increased

consumption, bingeing, withdrawal, craving, and cross-sensitization to other rewards (Avena et al., 2008; Corwin et al., 2011). Natural rewards and many drugs of abuse act on a common or at least largely overlapping neural system. Similarly, behavioural addictions and drug addictions share many neural and behavioural commonalities. Taken together, this suggests that an improved understanding of one can inform the other.

To label sugar or food as addictive may be misleading because simple availability and consumption of palatable foods does not necessarily cause addiction-like behaviour. Recently, the ICP was used to explore whether the protocol produces any addiction-related changes other than increased consumption (Rehn & Boakes, 2019). Rats were given E4D vs. ED access to 4% solution in Phase I (28 days) and shifted to E2D access continued with 4% in Phase II (28 days). Next, rats were tested for various addiction-like behaviours and showed no evidence of “craving” in preference tests or “withdrawal” in the anxiety test (elevated-plus maze) in their “binge” group (the E3D group in the ICP). Rhen and Boakes (2019) suggested the addiction-like behaviours that have been found in other intermittent access protocols may not exist under the more controlled and circadian-independent conditions like with the ICP.

For sugar consumption to be considered excessive, it can be argued these rats should gain more weight than rats fed chow only, ultimately leading to obesity. With intermittent access protocols, rats maintain homeostatic caloric intake despite showing elevated levels of sucrose intake (Eikelboom & Hewitt, 2016; Avena et al., 2008, Corwin, 2011). On days when sucrose is available,

both ED and E3D groups reduce food and water intake, and the sucrose solution consumed seems to be a redeployment of the typical amounts of food and water consumed by rats. This pattern is evident in the ED groups, and much more pronounced in the E3D groups (Hewitt & Eikelboom, 2016). Rather than the term "sugar addiction", it may be more accurate to describe the increased consumption of sugar (and related changes) observed in rats with intermittent access to sucrose, as an acquired increased motivation for sweets, or "sweets-motivation".

The reason rats increase their intake of a palatable food or drink when it is only available intermittently, and the mechanisms that underlie this behaviour are not known; my work provides some contribution to this area. One possibility is that rats given intermittent access to specific food sources increase intake of these sources because of the uncertainty associated with their availability (Corwin, 2011). The large difference we typically find with adults given 4% E3D vs. ED reflects reduced intake by the continuous group as well as increased intake over time in intermittent rats. The latter, possibly due to the uncertainty with availability. Perhaps with adults, 4% is an ideal solution to show this difference, as weaker solutions do not provide a strong enough reward, and stronger solutions limit intake because of the caloric load. For the same reason (caloric limit), we might not see a difference in pups with 4%, thus, testing pups with 2% in Phase I might be helpful. Because 2% is less rewarding than 4%, and pups did not develop the Phase I or Phase II (longer-term) difference with 4%, we might not expect pups to develop the longer-term difference with a weaker 2% solution. To better

understand the results in pups, testing pups with weaker solutions (1%, 2%) with addition of artificial sweeteners to increase the hedonic value of weaker solutions without impacting caloric load of the solutions might be helpful. Work by Folmer (2020) suggests that calories are more important than taste for the development of the ICP effect, as adult rats given 4% + saccharin solution (isohedonic to 12% sucrose) show consumption patterns similar to rats given 4%, rather than rats given 12%. Thus, it might also be more important to focus on testing pups with stronger solutions than 4%. Testing pups with the ICP and solutions stronger than 4% could determine the minimal strength at which pups develop the longer-term change.

Relevance of this Rat Work to Humans

Much like the work of most behaviourists (Amsel 1994; Blodgett, 1929; Skinner, 1954; Stewart et al., 1984), our rat work with the ICP is not primarily focused on understanding species-specific animal behaviour, like an ethologist, but more broadly as well, as an experimental model for exploring and understanding human behaviour (Celejewski, 2011; Eikelboom & Hewitt, 2016, Senthinathan, 2012; Valyear, 2014).

The presentation of maladaptive consumption disorders in humans shows a developmental trajectory, with less reports in children and more in adolescents (Schramm-Sapyta et al., 2009). Work with the 12h-12h, M-W-F, and ICP have directly aimed to make connections with human behaviour (Avena et al., 2008; Corwin & Babbs, 2012; Rhen & Boakes, 2019). Most intermittent access rat protocols focus on binge-behaviour and might be relevant to the human binge

eating pattern of avoiding certain types of food (typically high-fat or sugary items) and then consuming them excessively. Of the three intermittent access protocols noted above, it could be argued the M-W-F and ICP are more relevant to human binge behaviour because these two do not involve any food or water restriction while the 12h-12h protocol involves restriction, so at least in these two, rats are not increasing intake because of hunger or thirst, as with human binge eating (Balantekin et al., 2017). Bingeing has been reported in intermittent groups with the M-W-F (Corwin & Babbs, 2012) and the ICP (Rhen & Boakes, 2019).

Intermittent access might be considered a “less structured” and less predictable environment vs. continuous access, which is more structured and more predictable, because of the uncertainty associated with intermittent access (Woods, 1991). The binge-like behaviour with intermittent protocols and increased daily intake by intermittent vs. continuous groups with the ICP, might be related to the predictable nature of a continuous availability, compared to the less predictable nature of intermittent availability. When binge eating develops in humans, it typically gradually presents over adolescence. I found a similar pattern in rats with the ICP.

For most humans in developed countries the current environment is one of food abundance, which seems similar to continuous access at face value. The “unstructured food environment” shared by most people in communities with food abundance (e.g. irregular meal timing, Sisson et al., 2011; irregular/unpredictable meal location, Guthrie et al., 2002) suggests current human consumption patterns might be more related to the “less structured” intermittent environment.

Adolescents who typically eat dinner with their families were less likely to eat excessively compared to adolescents who typically do not eat with their families (Haines et al. 2010). Setting a regular eating schedule is effective in treating excessive (binge) eating in humans (Murphy et al. 2010). More structured environments might limit the development of excessive consumption and conversely, less structured food environments might increase the likelihood of developing excessive consumption.

Comparative investigation in rats and humans shows pups, and children, tend to prefer sweeter solutions than older rats (Bertino & Wehmer, 1981; Wilmouth & Spear, 2009), and humans (Drewnowski, 1989; 2000), respectively. Biological mechanisms strongly influence sucrose consumption patterns across species. Adolescents consume more sugar sweetened beverages than both adults and children (Langlois & Garriguet, 2011), and this pattern is possibly related to both an evolutionarily adaptive “developmental sweet tooth” as well as the increased freedom to choose food sources in adolescence compared to children.

Adolescents. As noted above, maladaptive consumption disorders are not typically reported in children; instead they seem to first present during adolescence (Schramm-Sapyta et al., 2007). I found similar results in rats. Pups did not show differences in Phase I with either concentration, even though the intermittent vs. continuous experience with 16% had a profound influence on their behaviour, which emerged later. All of the pup effects found in my dissertation were strictly observed during adolescence. Extrapolating, latent

maladaptive consumption disorders might develop in children and not present until later on, ultimately contributing to the peak in human binge behaviour associated with adolescence (Marzilli et al., 2018). Another age-parallel with my rat work, human adolescents consume more sugar than children and adults (Langlois & Garriguet, 2011), and adolescent rats consumed more sucrose than other age groups (Chp. 2, Exp. 1).

Adolescence is characterized by an increased propensity towards risky behaviour and development of drug dependence (Bernheim et al., 2013). Initiation of drug use during this period increases the likelihood of life-long addiction-related issues (Chambers et al., 2003; Grant & Dawson, 1998; Jordan & Anderson, 2017; Wagner & Anthony, 2002). Persistently increased/excessive sucrose intake in the face of frequent availability might be considered a maladaptive behavioural change. One important difference is that individuals typically learn to consume sugar immediately after birth but are typically only exposed to drugs of abuse later in development.

Adolescence is the developmental period during which mammals typically begin to venture away from the home and become independent from their parents. Extreme vigilance to ecological context and ongoing environmental changes during adolescence is critical, as it could increase an organism's chance of survival. For example, in an environment that involves periods of food abundance followed by longer periods of scarcity, it would make sense for an organism to develop a binge-like consumption strategy to consume as much nutrition as possible whenever food is available. This sort of mechanism that is

tuned to uncertainty of rewards would be adaptive for organisms that are responsible for their own food collection and might explain why adult rats are also profoundly influenced by the ecological context of intermittent sugar availability. It has been suggested the adolescents are particularly vulnerable to the influence of environment on reward-related behaviour, because during this developmental period, organisms undergo significant neural development and reorganization in neural areas associated with motivation and reward (Spear, 2000). Perhaps one reason why adolescence is touted as a particularly vulnerable time in development is because of its contrast to the pup period, which is marked by a high level of parental dependence.

Children. There is considerably less literature exploring the development of consumption related disorders in children compared to the adolescent period. Childhood in humans is a maturational stage suggested to be associated with invulnerability to disorders of consumption (Jordan & Anderson, 2017). Previous work suggests that childhood exposure to stimulants reduces the rewarding properties of these drugs (Biederman et al., 1999; Mannuzza et al., 2008; Wilens et al., 2003). Furthermore, childhood exposure to stimulant drugs has been suggested to have a protective effect against the development of substance use disorders (Jordan & Anderson, 2017). Thus, it seems that children may be protected from developing maladaptive consumption patterns. As childhood in humans coincides with the pup phase in rats, my work might suggest otherwise. I found pups given intermittent vs. continuous access to sucrose can develop, at older ages, persistent behavioural differences. The type of intake difference that

developed in pups (but was latent) and showed later in adolescence has been described as sucrose bingeing in the intermittent groups compared to the continuous groups (Rehn & Boakes, 2019). Thus, it may be argued that based on my findings, pups can develop the propensity to binge-behaviour.

Children are often provided with sugary and highly-palatable foods as a reward. This is in contrast to other, less palatable foods that are likely more regularly available. Since children are more likely to be given sugary foods intermittently, rather than continuously, it may be important to further explore how repeated periods of intermittent access to sweets during childhood in humans, or as pups in rats, contributes to consumption behaviour in the short- and longer-term.

Conclusion

It is difficult to disentangle the contribution of social and biological factors to the development of consumption disorders (Schulte et al., 2017; 2018). My work is part of a long lineage of research that has demonstrated the power that simple changes in environment can have on behaviour and supports that overconsumption may be a learned behaviour. My data shows that very early in development individuals may be less vulnerable to the influence of poor environment or reward-uncertainty, but there is a threshold to this effect. By increasing the rewarding value of the infrequent reward, very young rats learned to consume elevated amounts of sucrose, which became evident later in life. Extrapolating to humans, this might suggest that children are also not

invulnerable to the influence of environment on longer-term patterns of reward consumption. The supposition that maladaptive consumption behaviours can develop in children but remain “hidden” until much later in adolescence is particularly concerning. There is a need for comparative animal studies to address how developmental changes influence the way rewards are consumed. By targeting early developmental periods, future comparative animal research can uncover the evolutionary mechanisms that influence reward related consumption patterns, as well as how these mechanisms change with maturation.

Appendix A

Table A1. Weight data for all rats on all common sucrose days (Chapter 2, Experiment 1).

Age Group	Access Group	Days of age:	22	25	39	42	56	59	76
Pups	ED		49	74	175	197	322	352	503
			54	79	202	225	350	382	530
			49	69	185	206	320	346	499
			51	73	191	217	336	368	524
			52	69	192	219	331	355	510
			48	70	181	202	310	336	479
			48	76	183	204	311	334	467
			47	68	176	200	313	337	478
	4D		50	77	206	235	359	389	550
			50	72	186	206	317	340	482
			51	70	186	211	330	358	503
			53	78	198	224	341	371	524
			54	70	179	197	305	327	460
			50	73	188	209	345	372	537
			43	66	166	191	299	323	468
			52	68	193	216	333	364	502
	20D		51	77	192	217	339	368	514
			49	71	177	201	311	332	468
			51	70	181	199	318	351	505
			51	67	184	210	324	348	506
			43	69	167	190	294	322	460
			40	69	158	179	299	330	468
			47	76	174	196	291	318	445
			52	67	203	236	355	381	548
Average for Pup Groups			**49.35**	**71.67**	**184.30**	**207.79**	**323.04**	**350.17**	**497.04**
Adolescents	ED		50	77	187	210	323	351	501
			50	73	187	211	316	346	490
			50	70	193	226	364	390	581
			50	68	171	192	301	325	479
			48	70	180	201	311	336	490
			49	69	184	211	334	363	507
			46	77	194	219	360	386	553
			48	68	174	190	301	327	452
	4D		52	75	194	217	327	351	500
			49	71	176	199	313	342	477
			50	69	176	201	296	321	449
			45	67	181	206	310	337	475
			49	68	196	223	333	367	484
			49	69	181	199	317	341	484
			49	76	166	181	284	301	428
			53	68	193	214	331	356	491
	20D		48	77	176	199	314	339	491
			53	73	206	230	351	377	537
			48	70	186	206	299	316	420
			48	69	189	220	345	375	525
			46	70	181	207	301	330	470
			50	69	195	216	353	381	560
			55	77	206	226	355	390	557
			47	68	181	199	320	346	512
Average for Adolescent Groups			**49.28**	**71.15**	**185.52**	**208.46**	**323.29**	**349.75**	**496.32**
Adults	ED		51	77	186	210	324	357	499
			55	71	189	215	288	304	397
			44	69	190	214	329	351	496
			50	67	180	201	313	344	489
			49	69	187	197	333	363	504
			48	69	194	221	351	382	538
			51	76	181	199	310	334	490
			50	67	184	212	322	342	480
	4D		55	75	226	259	380	415	555
			53	70	180	197	281	300	421
			50	69	188	214	333	363	497
			45	65	161	183	245	259	351
			45	68	160	181	271	296	421
			52	71	184	201	325	354	498
			53	75	200	219	351	379	545
			47	68	177	196	315	346	487
	20D		57	77	204	231	331	358	491
			51	72	185	206	325	353	495
			50	70	192	219	328	349	487
			45	68	181	200	330	360	514
			50	70	171	194	285	303	437
			50	70	188	209	314	338	485
			50	76	192	217	336	369	536
			45	67	176	196	309	341	493
Average for Adult Groups			**49.76**	**70.66**	**185.60**	**207.96**	**317.88**	**344.17**	**483.54**

Figure A1

Mean (± SEM) 4% sucrose solution intake (g) for all pup, adolescent, and adult groups (every day, intermittent 4D, intermittent 20D).

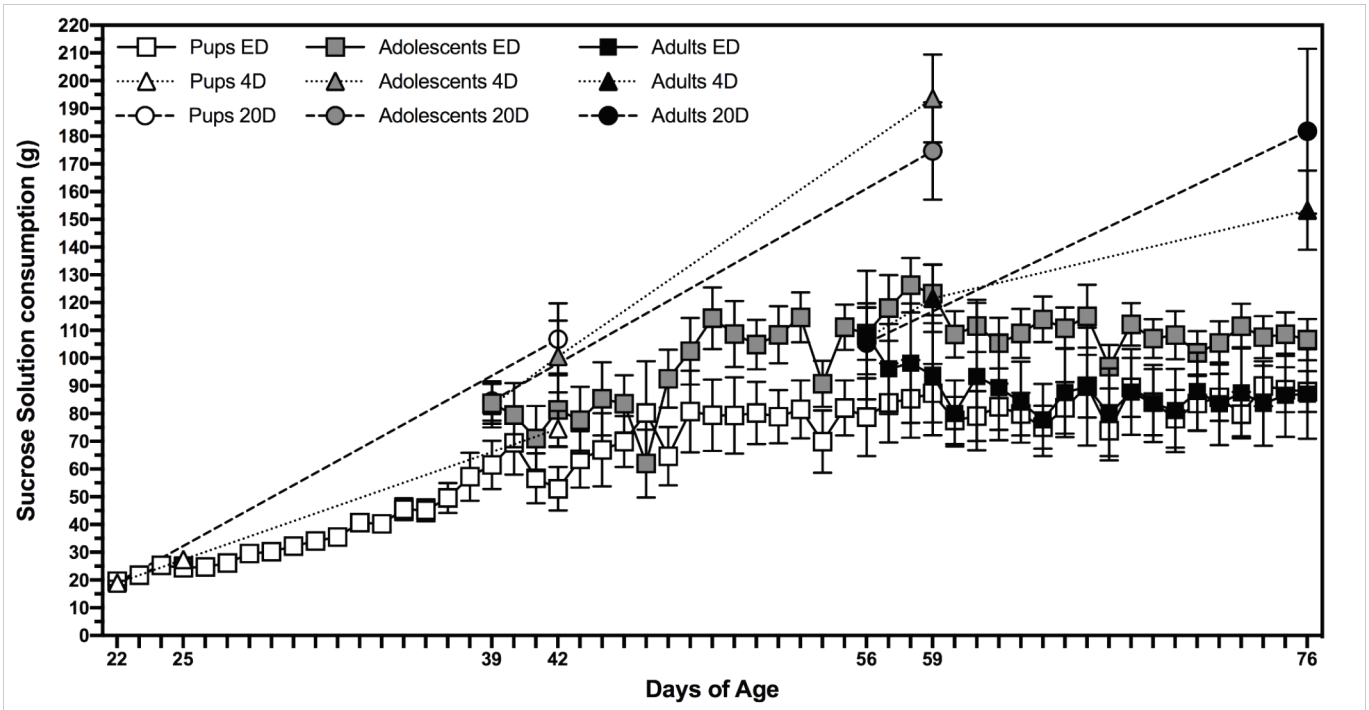
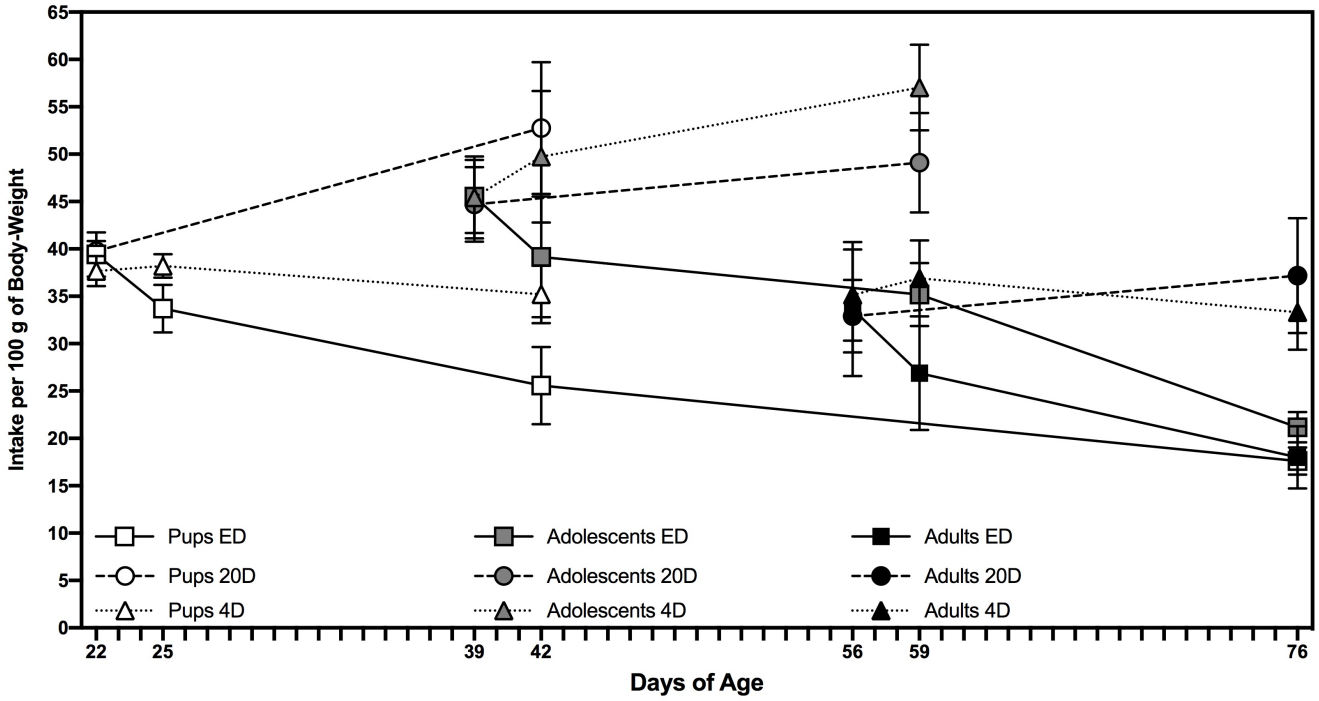


Figure A2.

Mean (± SEM) 4% sucrose solution per 100 g of body-weight for all pup, adolescent, and adult groups (every day, intermittent 4D, intermittent 20D).



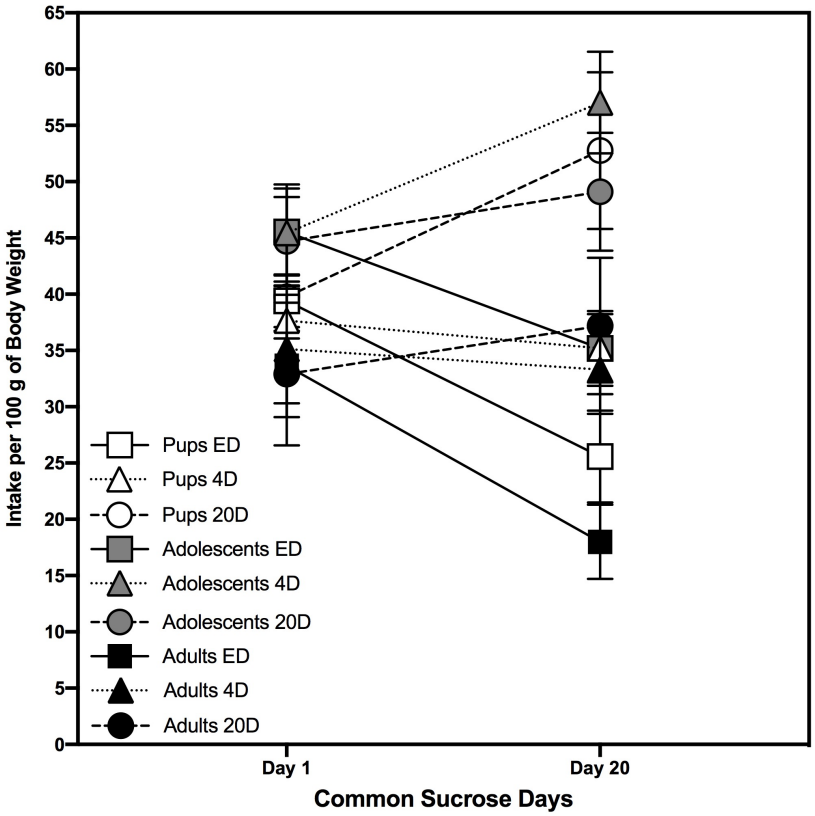
Appendix B

Sucrose Intake per 100 g of Body-Weight on Day 1 and Day 20 in all Groups

Mixed ANOVA comparing Day 1 and Day 20 consumption for all ages and all three access conditions was completed (Figure B1, below). In this ANOVA there was a significant Age effect ($F(2,63) = 11.33$, $p < .001$, $\eta_p^2 = .265$), but no interactions involving Age suggesting that as in the previous Day 1, 4, and Day 1, 20 comparisons, adolescent rats consumed more sucrose in all situations than pup and adults. There was also a significant Access effect ($F(2,63) = 5.81$, $p = .005$, $\eta_p^2 = .156$), but there was also a Day by Access interaction ($F(2,63) = 16.69$, $p < .001$, $\eta_p^2 = .346$). From Figure B1 it is evident that while consumption dropped from Day 1 in the ED group rats it increased in the 4D group, and 20D group rats. Subsequent single day ANOVAs of these 6 groups revealed, as expected, only an Age difference on Day 1 ($F(2,63) = 5.94$, $p = .004$, $\eta_p^2 = .159$), with adolescent rats consuming more than rats at the other ages. On Day 20 there were significant Age ($F(2,63) = 10.66$, $p < .001$, $\eta_p^2 = .253$), and Access ($F(2,63) = 15.29$, $p < .001$, $\eta_p^2 = .327$), main effects but no significant interaction suggesting that the three ages had similar access induced changes. At all ages the consumption was lower in the continuous access conditions and higher in the intermittent access conditions with adolescent rats generally having a higher consumption than the other two groups.

Figure B1

Mean (± SEM) sucrose intake per 100 g of body-weight on Day 1 and Day 20 by pup, adolescent, and adult every day (ED), intermittent 4D, and intermittent 20D groups.



Appendix C

Table C1.

Bregma coordinates.

Brain Region	Bregma
Caudate-Putamen (Central)	2.2
Caudate-Putamen (Dorsal)	2.2
Caudate-Putamen (Dorsolateral)	2.2
Caudate-Putamen (Medial)	2.2
Dorsal Endopiriform Nucleus	2.2
Dorsal Tenia Tecta	2.2
Insular	2.2
Islands of Calleja	2.2
Nucleus Accumbens Core	2.2
Nucleus Accumbens Shell	2.2
Piriform	2.2
Ventral Pallidum	2.2
Bed Nucleus of the Stria Terminalis (Lateral)	0.2
Bed Nucleus of the Stria Terminalis (Medial)	0.2
Bed Nucleus of the Stria Terminalis (Ventral)	0.2
Lateral Septum (Dorsal)	0.2
Lateral Septum (Intermediate)	0.2
Lateral Septum (Ventral)	0.2
Cingulate	0.2
Paraventricular Hypothalamic Nucleus Lateral Magnocellular Part	-1.8
Paraventricular Hypothalamic Nucleus Dorsal Cap	-1.8
Paraventricular Hypothalamic Nucleus Medial Parvicellular Part	-1.8
Paraventricular Hypothalamic Nucleus Ventral Part	-1.8
Supra Optic Nucleus	-1.8
Central Nucleus of the Amygdala	-2.3
Cingulum	-2.3
Ventromedial Hypothalamic Nucleus	-2.3
Paraventricular Thalamic Nucleus	-3.0
Subthalamic Nucleus	-3.8
Supramammilary Nucleus	-4.8
Ventral Tegmetal Area	-4.8
Edinger-Wesphal Nucleus	-5.28
Substantia Nigra Compacta	-5.28
Substantia Nigra Lateral	-5.28
Substantia Nigra Reticular	-5.28
Periacquaductal Grey (Dorsal)	-7.8
Periacquaductal Grey (Dorsolateral)	-7.8
Periacquaductal Grey (Lateral)	-7.8
Periacquaductal Grey (Ventrolateral)	-7.8
Locus Coereleus	-9.72

Note. The order of brain areas corresponding with the y-axis (top down) of the heat plots (Figure 4.5). The brain regions are arranged rostrocaudally based on the bregma coordinates from which data was collected.

Appendix D

Table D1. Mean Fos counts in 40 brain regions (Chapter 4, Experiment 1).

		Pup 4% ED		Pup 4% E3D		Pup 16% ED		Pup 16% E3D		Adult 4% ED		Adult 4% E3D		Adult 16% ED		Adult 16% E3D	
Brain Region	***Subsection***	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Cortex																	
Insular cortex		5.8	2.1	3.8	1.7	3.4	1.3	4.4	1.8	2.1	0.9	3.6	1.2	4.4	2.3	5.1	2.0
Cingulate cortex		3.3	1.3	3.4	0.9	2.3	0.7	1.8	0.8	2.5	1.1	3.1	1.2	5.7	1.6	3.2	1.1
Piriform cortex		4.1	1.4	3.8	1.0	2.6	0.9	2.8	0.7	3.8	1.0	4.9	2.1	3.8	1.3	4.6	2.0
Dorsal lenis fascia		9.8	1.1	9.3	1.7	8.5	1.6	7.1	1.3	7.8	2.7	10.1	2.5	8.5	1.9	9.0	3.7
Central nucleus of the amygdala		1.2	0.6	0.6	0.3	2.6	2.3	0.6	0.4	0.6	0.4	1.6	0.8	0.4	0.2	0.6	0.1
Subcortical																	
Bed nucleus of the stria terminalis	*lateral*	2.8	1.3	4.6	0.8	4.2	1.2	5.1	2.0	2.8	1.1	3.2	0.8	1.8	0.5	3.4	0.9
	medial	4.7	1.1	6.3	1.3	4.8	1.3	4.9	1.4	6.3	2.1	5.3	1.2	4.4	0.9	5.1	1.1
	ventral	7.6	2.2	7.5	1.8	7.7	2.0	7.4	2.2	6.3	1.5	6.3	1.5	6.9	1.1	6.5	0.9
Cingulum		0.4	0.2	0.4	0.3	0.1	0.1	0.1	0.1	0.3	0.3	0.8	0.8	1.1	0.7	0.8	0.5
Dorsal endopiriform nucleus		2.2	1.0	4.4	1.5	3.0	1.4	2.9	1.1	3.1	0.9	2.9	0.9	2.8	1.2	3.9	1.7
Diencephalon																	
Ventromedial hypothalamic nucleus		6.5	2.0	5.3	2.4	6.4	2.4	6.1	2.7	4.0	1.3	5.1	1.6	5.6	1.5	3.6	0.8
Periventricular hypothalamic nucleus	*lateral magnocellular part*	2.1	0.9	2.8	0.9	1.7	0.5	1.8	0.9	0.3	0.2	2.6	1.0	2.1	1.0	0.9	0.4
	dorsal cap	3.0	1.6	5.6	0.6	4.6	1.3	4.3	2.3	1.2	0.4	3.8	1.2	3.9	1.5	2.1	0.5
	medial parvicelular part	4.7	2.0	6.5	1.5	5.1	1.6	4.2	1.6	3.7	0.9	7.4	1.8	5.9	2.0	3.6	0.6
	ventral part	7.8	1.6	8.3	3.0	6.7	1.6	7.3	2.4	6.3	1.3	5.0	1.1	7.2	1.8	5.8	1.2
Supraoptic nucleus		1.3	0.9	0.6	0.4	0.9	0.5	0.6	0.2	1.1	0.6	1.4	0.5	1.0	0.5	1.9	0.7
Supramammillary nucleus		15.2	5.0	9.4	2.5	10.3	2.6	8.9	2.0	7.4	1.6	5.5	0.5	10.8	2.4	7.0	0.8
Paraventricular thalamic nucleus		6.9	2.3	17.4	2.3	15.1	2.9	11.9	3.7	8.4	1.9	16.4	2.0	13.8	2.4	15.6	2.2
Basal ganglia																	
Caudate putamen	*central*	0.8	0.6	0.3	0.1	0.6	0.4	0.6	0.3	0.0	0.0	0.3	0.3	0.4	0.3	0.6	0.4
	dorsal	0.7	0.4	0.3	0.1	0.4	0.2	0.9	0.4	0.4	0.3	0.4	0.4	0.3	0.3	0.3	0.2
	dorsolateral	2.3	0.9	0.4	0.4	0.6	0.5	0.6	0.3	0.7	0.5	0.9	0.6	0.6	0.6	0.9	0.6
	medial	0.4	0.3	0.9	0.8	0.9	0.5	0.6	0.5	0.8	0.7	1.1	1.1	1.0	0.7	0.3	0.2
Islands of calleja		0.3	0.2	0.6	0.2	0.2	0.1	1.1	0.6	0.8	0.5	1.4	0.7	0.2	0.1	0.6	0.4
Nucleus Accumbens	*core*	1.6	0.7	1.6	1.0	2.8	1.9	3.6	2.0	2.3	1.0	1.6	1.0	3.4	1.7	1.9	0.8
	shell	1.9	1.1	1.6	0.8	3.9	1.1	3.6	1.4	1.3	0.6	1.1	0.8	2.1	1.2	0.9	0.7
Substantia nigra	*compacta*	0.1	0.1	0.3	0.1	0.4	0.2	0.0	0.0	0.2	0.1	0.4	0.2	0.6	0.3	0.3	0.2
	lateral	1.7	0.7	3.4	1.1	3.9	1.0	1.4	0.7	3.1	1.2	2.3	1.2	3.9	1.1	2.8	0.7
	reticular	1.1	0.7	1.8	0.9	1.9	0.7	1.0	0.5	1.1	0.6	0.4	0.4	0.8	0.6	1.4	0.5
Subthalamic nucleus		0.9	0.5	1.0	0.5	1.1	0.8	0.6	0.5	0.4	0.2	0.4	0.4	1.3	0.6	1.1	0.6
Ventral pallidum		2.7	0.7	2.9	0.9	1.3	0.8	3.1	1.0	3.3	1.3	1.7	1.0	3.4	1.1	1.3	0.8
Ventral tegmental area		2.4	0.5	1.1	0.5	3.1	1.5	2.4	0.9	1.1	0.6	1.9	0.5	3.8	1.0	1.7	0.5
Brainstem																	
Edinger-Westphal nucleus		1.9	0.6	1.1	0.7	0.9	0.4	1.6	0.9	1.4	0.7	2.4	0.6	2.6	1.3	1.4	0.7
Locus coeruleus		2.2	1.3	2.3	0.7	2.0	0.6	0.8	0.5	1.0	0.5	3.3	1.7	2.0	0.6	1.4	0.8
Lateral septal nucleus	*dorsal*	1.4	0.9	1.8	0.5	1.9	1.0	1.4	0.9	3.2	1.0	2.6	0.3	2.9	1.2	1.1	0.3
	intermediate	1.3	0.7	2.9	0.9	2.8	0.8	1.2	0.8	4.4	1.8	2.6	1.0	3.1	1.1	1.6	0.7
	ventral	3.3	1.4	7.1	2.2	7.8	1.5	4.1	1.7	4.8	1.6	7.2	1.4	8.7	1.7	3.9	0.6
Periaqueductal gray	*dorsal*	1.0	0.6	2.0	0.6	1.1	0.4	2.1	0.8	1.1	0.6	3.4	1.9	2.5	0.9	0.9	0.3
	dorsolateral	1.7	0.7	1.4	0.6	1.6	0.4	2.4	1.5	1.1	0.7	2.2	0.6	2.8	0.8	1.6	0.5
	lateral	1.3	0.5	1.6	0.7	2.3	0.7	1.4	0.8	1.3	0.6	1.0	0.4	0.9	0.6	1.3	0.4
	ventrolateral	2.8	1.1	3.5	1.1	4.1	1.1	3.4	1.2	1.1	0.4	4.7	1.5	2.7	1.1	3.0	1.4

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